THE INTERACTIONS OF SURFACE CARBOHYDRATES OF *MELOIDOGYNE* SPP. WITH SOYBEAN ROOTS

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Fluorescent (rhodamine) conjugates of the lectins, soybean agglutinin (SBA), Concanavalin A (CON A), wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LOT), and *Limulus polyphemus* agglutinin (LPA) bound exclusively to amphidial openings and cephalic secretions of preinfective second-stage juveniles (J2) of *Meloidogyne incognita* races 1 and 3 (Mi1, Mi3), and *M. javanica* (Mj). No substantial differences in fluorescent lectin-labeling were observed among preinfective J2 of the *Meloidogyne* spp. populations examined, and only binding of LOT and LPA was inhibited in the presence of 0.1 M competitive sugar. Differences in structure of amphidial carbohydrate complexes among populations of Mi1, Mi3, and Mj were revealed by glycohydrolase treatment of preinfective J2 and subsequent labeling with fluorescent lectins. Several glycohydrolases

eliminated binding of LPA to the amphidial region of J2 of the Meloidogyne spp. tested. Quantitative differences in binding of the peroxidase-labeled lectins, SBA, CON A, LOT, WGA, and LPA to J2 of Mi1, Mi3, and Mj were determined by microfiltration enzyme-linked lectin assay. Preinfective J2 of Mj bound the greatest amount of SBA, LOT, and WGA, whereas preinfective J2 of Mi1 bound the most LPA in two separate experiments. Preinfective J2 of Mi3 generally bound the least amount of all lectins tested.

Treatment of J2 of Mi1 and Mj with purified, unconjugated SBA, CON A, WGA, LOT, or Limax flavus agglutinin (LFA) did not influence root tissue response of 'Centennial' and 'Pickett 71' soybean cultivars to infection by Mi1 or Mj. Giant cells were usually associated with untreated Mi3 in Centennial root tissue 20 days after inoculation. Treatment of J2 of Mi3 with lectins or carbohydrates caused Centennial root tissue to respond to infection by treated Mi3 in a hypersensitive manner. Nematodes could not be detected within soybean roots 5 days after inoculation of root tips with J2 suspended in solutions of LFA or sialic acid. Only treatment of J2 with sialic acid and sialic acid plus LPA strongly reduced reproduction of all populations of Meloidogyne spp. in soybean roots of both cultivars. Treatment of J2 of Meloidogyne spp. with LFA, LPA, or sialic acid was not lethal to nematodes.

CHAPTER 1
INTRODUCTION

Most agricultural crops are vulnerable to attack by phytoparasitic nematodes. Nematode damage has caused estimated losses as high as 21% in major U.S. crops (111). Dowler and Van Gundy (26) recently estimated annual world crop losses to nematode damage to be in the order of $500 million. Root-knot nematodes (Meloidogyne spp.) are considered to be the most economically important phytoparasitic nematodes because of their world-wide distribution, interaction with other phytoparasitic organisms, and extensive host range (100). The host range includes almost all of the plants that account for the majority of the world's food supply (100, 101, 102, 121). Four major species of root-knot nematode, Meloidogyne incognita (Kofoid and White) Chitwood, Meloidogyne javanica (Treub) Chitwood, Meloidogyne hapla Chitwood, and Meloidogyne arenaria (Neal) Chitwood comprised over 95% of the root-knot nematode populations detected in over 500 samples collected from agroecosystems around the world (100).

The principal management strategies implemented to reduce nematode-related crop damage include field application of fumigant and nonfumigant nematicides, cultivation of plant varieties which are resistant to phytoparasitic nematodes, and prudent cultural practices such as sanitation and crop rotation. Nematicides are relied upon as the primary means of reducing nematode-related crop losses in many crops. Their toxic properties, water solubility, and persistence in the environment have, however, triggered restriction of nematicide application, making them unavailable for use or reducing their efficacy in many economically important crops (54). Nematicides often cannot be incorporated into agricultural pest management strategies in developing nations because chemicals may be inaccessible or too expensive, application equipment may not be available, and growers may not be properly educated in the safe and effective use of pesticides (18, 101). To date, crop rotation is still the most widely used pest management strategy implemented on a world-wide basis (41, 73). Difficulties encountered in crop rotation schemes include identification of potentially damaging phytoparasitic nematodes and their host ranges, selection of suitable nonhosts for polyspecific nematode field populations or species with wide host ranges, and

production of crops that are economically beneficial for growers and that are marketable (41).

Cultivation of crops that are resistant to diseases induced by root-knot nematodes is appealing because it provides an effective, economical, and environmentally safe means of reducing nematode-related crop damage (32, 33). Hundreds of crop cultivars are currently available that possess resistance to one or more species of root-knot nematode (32, 33, 103). Resistance is a term that encompasses two main components: the ability of a plant to tolerate nematode-related damage or to limit nematode reproduction (58, 59). Thus, varying degrees of resistance to phytoparasitic nematodes have been reported among different plant cultivars (34). Most nematode-resistant germplasm has been developed towards nematodes that are endoparasites because, according to Roberts (94), "natural selection of resistance genes is more likely to have occurred in the most highly specialized host-parasite relationships where co-evolutionary development of host and parasite has produced a highly specific interaction in which host and parasite compete for a genetic advantage (118)."

This dynamic interaction between host and parasite on a microevolutionary scale threatens the durability of resistance in plants to nematodes. Continued cultivation of nematode-resistant crops on the same parcel of land has

selected for populations of phytoparasitic nematodes that overcome plant resistance to nematode attack (34, 99, 108, 119, 123). Although the genetic basis of inheritance of resistance to nematodes has been identified for a number of plant cultivars (9, 34, 55, 108), little is known about how resistance genes function (58). A greater knowledge of the mechanisms of plant resistance to nematodes should facilitate the application of germplasm modification and the development of bioengineering for the transfer of nematode resistance genes between plant genomes (34, 58, 71).

A number of studies have investigated the mechanisms of plant resistance to nematodes and the results have been discussed in several reviews (37, 39, 42, 58, 59, 97, 127). The terms "incompatible" and "compatible" are used here to designate plant-nematode interactions that inhibit nematode development and reproduction, and interactions which promote nematode development and reproduction in plants, respectively (58, 59). Passively or preinfectionally incompatible plant-nematode interactions may involve morphological plant barriers to nematode infection or constitutive plant factors that affect egg hatch, locating a food source, survivability, and host suitability (32, 35, 39, 58, 59, 97, 127). Actively or postinfectionally incompatible plant-nematode interaction involves the elicitation and subsequent sequence of host defense

reactions induced by challenges of certain nematode species or races to specific plant cultivars (32, 58, 59, 127).

The physiological sequence of events following elicitation of active plant defenses, the "expressive phase" (63), has been described for a number of incompatible plant-nematode interactions (58). Inhibition of nematode development in incompatible plant cultivars is often associated with hypersensitive reactions (HR) in plant tissue adjacent to nematodes and in feeding sites shortly (hours) after nematode or stylet penetration of roots (12, 43, 57-59, 109, 112, 134). Phytoalexin accumulation has been associated with the HR in some incompatible plant-nematode interactions (43, 60, 92, 130), and the potential involvement of phytoalexins in incompatible plant-microbe interactions has been discussed (7, 42, 59, 68, 128).

The incompatible and compatible interactions of soybean (Glycine max (L.) Merr.) cultivars with root-knot nematodes have been examined in detail (28, 30, 56, 60, 61, 129). The introduction and incorporation of genes for resistance to root-knot nematodes, primarily M. incognita, into successive soybean cultivars and the development of soybean resistance-breaking populations of Meloidogyne spp. have been summarized (33, 105). Resistance to M. incognita in soybean is conditioned by one major gene with at least one modifying gene (16, 98), and the degree of resistance

varies with the soybean cultivar and the population of M. incognita examined (8, 10, 15, 65, 96). The tissue reaction of roots of two related soybean cultivars to infection by the same population of M. incognita has been examined progressively from one to twelve days after exposure of soybean root tips to second-stage juveniles (J2) of M. incognita (61). Giant cell formation in 'Pickett 71' soybean roots infected with M. incognita (compatible) progressed normally throughout the observation period. The incompatibility of 'Centennial' soybean with M. incognita was associated with an HR of soybean root tissue in the region of invading J2 within 3 days of inoculation. The HR was strongly correlated with the accumulation of the phytoalexin, glyceollin, but biotic elicitors of the HR were not identified (60).

Indeed, little is known about the "determinative phase" (63) of plant-nematode incompatibility; factors are involved in this phase that enable an incompatible plant cultivar to recognize a specific potential pathogen and invoke active plant defenses (58). Since nematodes locate and penetrate roots of most cultivars in either compatible or incompatible pathosystems, recognition of endoparasitic nematodes by incompatible plants appears to occur following penetration (1, 24, 44, 90, 116, 124). The primary determinants in the specificity of plant-nematode

incompatibility probably include the interactions of nematode and plant cell surfaces, nematode secretions, and derepression of nematode and plant genomes (58).

The concept that recognition and specificity in plant-microbe interactions may involve the interaction of carbohydrate moieties of cell surface glycoconjugates and corresponding receptors on the surface of cells of which they come in contact, similar to cell to cell communication involved in antigenicity, blood group specificity, and mitogenesis in animal systems, was proposed by Albersheim and Anderson-Prouty (3). Numerous investigations of this hypothesis in different plant-microbe systems have been conducted and reviewed (21, 23, 31, 62, 83, 106, 136). The potential interaction of bacterial surface carbohydrates and lectins present on the surface of plant root cells as determinants of recognition and specificity in mutualistic Rhizobium spp.-legume interactions has been examined (6, 87, 126). For example, the binding of soybean and clover lectins appeared to be specific for most nodulating strains of Rhizobium spp. (13, 25). Some evidence for the involvement of cell surface interactions in incompatible plant-microbe interactions includes the elicitation of glyceollin accumulation in soybean tissue exposed to polysaccharide (4, 5) and glycoprotein-rich (64) wall fractions isolated from Phytophthora megasperma Drechs. f.

sp. glycinea, and induction of glyceollin accumulation in soybean tissue exposed to fractions of cellular envelopes isolated from incompatible races of Pseudomonas glycinea Coerper (19). Whether interaction of cell surface macromolecules is important in recognition and specificity in plant-microbe interactions remains controversial (6, 23, 87, 126). Inconsistencies in some results are difficult to interpret and may involve factors other than surface to surface interactions.

Characterization of the surface carbohydrate composition of a number of nematode species and the potential involvement of surface carbohydrate interactions in recognition and specificity between nematodes and other organisms have been investigated and are discussed in several reviews (47, 58, 80, 135, 136). Selected evidence from some of these investigations and information gathered since publication of the above reviews is mentioned in the introductions to the chapters within this dissertation. Zuckerman and Jansson (136) have postulated that specific interactions between nematodes and other organisms may be influenced by modification of nematode surface carbohydrates. The objectives of this investigation were to characterize the surface carbohydrates of second-stage juveniles of several populations of Meloidogyne spp. and to evaluate the potential involvement of nematode surface

carbohydrates in recognition and specificity in the incompatible and compatible response of two related soybean cultivars to infection by M. javanica and races 1 and 3 of M. incognita.

CHAPTER 2
CHARACTERIZATION OF CARBOHYDRATES ON THE SURFACE OF SECOND-STAGE JUVENILES OF *MELOIDOGYNE* SPP.

Introduction

The importance of surface carbohydrate biochemistry in recognition and specificity between plants and microorganisms has been the subject of many recent investigations and discussions (3, 21, 23, 31, 62, 106). Although few investigations concerning this phenomena have been conducted between nematodes and plants, surface carbohydrates of nematodes have been implicated in recognition between nematodes and nematophagous fungi (17, 47, 52, 80, 81, 135, 136). The surface carbohydrates of some helminth parasites of animals have been characterized and related to helminth antigenicity and chemoresponse (14, 72, 85). The involvement of surface carbohydrate recognition in the specificity of interaction between nematodes and *Pasteuria penetrans* Sayre and Starr has also been investigated (117).

Carbohydrates present on biological surfaces exist primarily as glycoconjugates such as glycolipids, polysaccharides, and especially as glycoproteins (79). The carbohydrate residues are often comprised of a number of

monosaccharide molecules covalently linked in various sequences and spatial arrangements (66, 122). The accessibility of surface carbohydrates to potential receptors in other organisms or as receptors of chemostimuli may be obscured by attached carbohydrate molecules, as is sometimes the case with sialic acids in animal systems (biological masks) (104). Enzymatic or inorganic chemical degradation can reveal "masked" carbohydrates that may exist on biological surfaces. Conversely, enzymes which cleave specific carbohydrate residues from glycoconjugates, glycohydrolases, can remove carbohydrates from biological surfaces and potentially alter biological interactions. An example of this latter phenomenon is the apparent loss of chemosensory perception of culture filtrates of Escherichia coli (Mig.) Castellani and Chambers by the nematodes Caenorhabditis elegans (Mau.) Dougherty and Panagrellus redivivus (L.) Goodey after treatment of these nematodes with mannosidase or sialidase (49).

Lectins, proteins that bind to specific carbohydrate residues, make excellent probes for the study of carbohydrates that exist on biological surfaces (31, 69, 70 107). Several methods, including lectin probes, have been used to characterize carbohydrates on the surface of a number of free-living and phytoparasitic nematodes, and the results of some of these investigations have been summarized

(47, 135, 136). Application of lectins to soil infested with Meloidogyne incognita (Kofoid and White) Chitwood reduced the number of nematode-induced galls on tomato roots, but the function of lectins in this system was unclear (74).

Several studies have attempted to relate nematode surface carbohydrates to specificity in plant pathogenicity (36, 76, 95). Differences in binding of fluorescent lectins to pathotypes of Globodera spp. and Meloidogyne spp. were reported (36, 76). The objective of this investigation was to characterize surface carbohydrates of three Florida populations of preinfective second-stage juveniles of Meloidogyne spp. using selected lectins and glycohydrolases.

Materials and Methods

Populations of Meloidogyne incognita races 1 and 3 (Mi1 and Mi3) and M. javanica (Treub) Chitwood (Mj) were maintained in greenhouse culture on roots of 'Rutgers' tomato (Lycopersicon esculentum Mill.) and 'Black Beauty' eggplant (Solanum melongena L.). Meloidogyne spp. populations were typified by adult female perineal patterns, second-stage juvenile (J2) lengths, and performance on differential hosts (101). Species identifications were also confirmed by three independent nematode taxonomists (A. M. Golden, Beltsville, MD; J. G. Baldwin, Riverside, CA; J. D.

Eisenback, Blacksburg, VA). Eggs of each nematode population were extracted from host roots with 0.53% NaOCl for 30 seconds (46) and hatched at room temperature on a Baermann funnel. Preinfective J2 that had hatched within 48 hours were used as test organisms in each experiment. A few eggs of Meloidogyne spp. were present in each suspension of J2.

Surface carbohydrates of preinfective J2

Fluorescent lectin probes were used to identify and locate carbohydrates on the surface of preinfective J2 of Mi1, Mi3, and Mj. Tetramethylrhodamine isothiocyanate (TRITC) conjugates of soybean agglutinin (SBA), wheat germ agglutinin (WGA), Concanavalin A (CON A), Lotus tetragonolobus L. agglutinin (LOT), and Limulus polyphemus L. agglutinin (LPA) (E-Y Labs, San Mateo, CA) were used. The ratios of absorbance at 550 to 280 nm for SBA, WGA, CON A, LOT, and LPA were 0.44, 0.55, 0.41, 0.57, and 0.20, respectively. The specific sugars to which each lectin binds are listed in Table 1-1.

A small sample of J2 (approx. 1000 J2) of each Meloidogyne spp. population was suspended in distilled water to serve as a control treatment. The remaining J2 of each population were concentrated into 2.0 ml of the appropriate buffer by centrifugation at 1000g for 3 minutes. Buffer solutions included: 0.01 M phosphate-buffer saline (PBS) at

Table 1-1. Sugar specificity and competitive sugars of soybean agglutinin (SBA), wheat germ agglutinin (WGA), Concanavalin A (CON A), Lotus tetragonolobus agglutinin (LOT), and Limulus polyphemus agglutinin (LPA).

Lectin	Sugar Specificity	Competitive Sugar[a]
SBA	α-D-galactose N-acetyl-α-D-galactosamine	D-galactose
WGA	N-acetyl-β-D-glucosamine	N-acetyl-D-glucosamine
CON A	α-D-mannose α-D-glucose	D-mannose
LOT	α-L-fucose	L-fucose
LPA	neuraminic (sialic) acid	N-acetylneuraminic acid

[a] Corresponding competitive sugars (0.1 M) used for all lectin and glycohydrolase assays.

pH 7.2 for SBA, WGA, and LOT; 0.05 M Tris-saline plus 0.01 M $CaCl_2$ at pH 7.5 for CON A; 0.05 M Tris-saline plus 0.01 M $CaCl_2$ at pH 8.0 for LPA. Preinfective J2 (approx. 5000 J2) of each population were incubated in lectin-TRITC conjugate (200 µg/ml) for 2 hours at 4°C. Additional treatments included nematodes (approx. 5000 J2) incubated in lectin-TRITC plus 0.1M corresponding competitive sugar (Table 1-1) to inhibit lectin binding, J2 incubated in 0.1M sugar plus buffer, and J2 incubated in buffer minus sugar. Treated J2 were washed by transferring J2 three times to microcentrifuge tubes that contained fresh buffer or water and allowing J2 to settle to the bottom of the tube. A sample of J2 (approx. 500 J2) in final wash solution was placed on a glass microscope slide and covered with a cover glass. The edges of the cover glass were sealed with clear fingernail polish. Approximately fifty specimens from each treatment were immediately observed at 100x under a Zeiss epifluorescent microscope equipped with TRITC and FITC (fluorescein isothiocyanate) filters. Photographs of selected nematodes were taken when nematode movement ceased (approx. 1-3 hours after J2 were mounted on slides). Each test was repeated twice.

The relative binding capacity of each lectin-TRITC conjugate was determined through hemagglutination assay (89). Twenty-five microliter volumes of lectin were

serially diluted (1:1) with the appropriate buffer in adjacent wells across a 96-well microtiter plate. Twenty-five microliters of a 4% suspension of trypsinized, gluteraldehyde-stabilized, human Type O red blood cells (Sigma Chemical Co., St. Louis, MO) were added to each well, except for wells containing LPA. A 4% suspension of gluteraldehyde-stabilized, horse red blood cells (Sigma Chemical Co.,St. Louis, MO) was used for LPA-TRITC assays. The greatest dilution of lectin that exhibited visible hemagglutination (titer) was determined after 3 hours incubation at room temperature. The titer divided by the milligrams lectin/ml in each sample is a measure of the specific hemagglutination activity of each lectin-TRITC conjugate. Similar tests were conducted in the presence of 0.1 M competitive sugar to assess inhibition of lectin binding activity.

Surface carbohydrates of glycohydrolase-treated preinfective J2

Enzymes (glycohydrolases) that cleave specific carbohydrate residues from glycoconjugates were assayed for their effect on surface carbohydrates of Mi1, Mi3, and Mj. Glycohydrolases tested consisted of the following: α-galactosidase (α-gal) EC 3.2.1.22 (20 U/mg) from recombinant E. coli, α-L-fucosidase (α-fuc) EC 3.2.1.51 (2.0 U/mg) from beef kidney, ß-N-acetyl-glucosaminidase (ß-glu)

EC 3.2.1.30 (4.0 U/mg) from beef kidney, α-mannosidase (α-man) EC 3.2.1.24 (10 U/mg) from Canavalia ensiformis DC., neuraminidase (sialidase) EC 3.2.1.18 (1.0 U/mg) from Clostridium perfringens (Veil. and Zub.) Holland. All glycohydrolases were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and enzyme activity was determined by reaction with the appropriate p-nitrophenyl conjugate of each carbohydrate substrate (information supplied by manufacturer).

Preinfective J2 of Mi1, Mi3, and Mj were each concentrated into 2.0 ml of the appropriate buffer by centrifugation at 1000g for 3 minutes. Buffer solutions included 0.01 M phosphate buffer (pH 7.2) for α-gal, 0.05 M sodium citrate buffer (pH 5.0) for α-fuc, 0.05 M sodium citrate buffer (pH 4.5) for ß-glu, 0.05 M sodium citrate buffer plus 1.0 mM $ZnSO_4$ (pH 4.5) for α-man, 5.0 mM sodium acetate buffer plus 72.0 mM NaCl and 7.0 mM $CaCl_2$ (pH 5.0) for neuraminidase. Enzyme buffers were formulated to the pH optimum of enzyme activity as suggested by the manufacturer. Preinfective J2 (approx. 5000 J2) of each population were incubated in either α-gal (1.0 U/ml), α-fuc (0.25 U/ml), ß-glu (1.0 U/ml), α-man (1.0 U/ml), or sialidase (0.25 U/ml) solution for 18 hours at 37°C. Nematodes were also incubated in enzyme plus 0.1 M corresponding competitive sugar (Table 1-1) to inhibit enzyme activity. Control

treatments consisted of J2 in buffer alone and J2 in buffer plus 0.1 M sugar at 37°C for 18 hours. Nematode viability after treatment with enzyme buffers under experimental conditions was confirmed by bioassay (Appendix B).

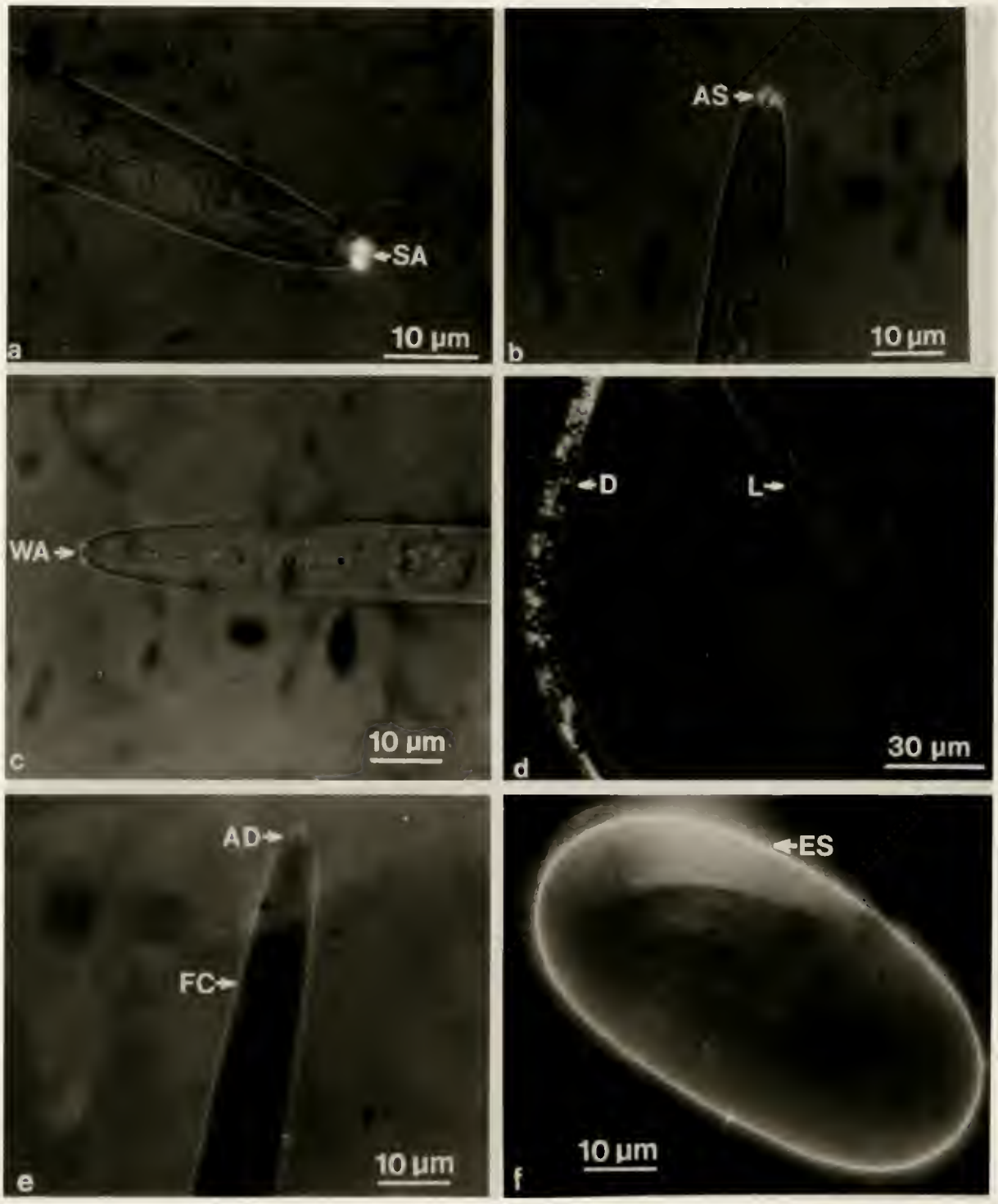
Glycohydrolase-treated nematodes were washed three times with the appropriate lectin buffer and subsequently treated with separate lectin-TRITC conjugates as described above for untreated, preinfective J2. Specimens were immediately mounted on glass microscope slides and observed under epifluorescent microscopy as described above.

Results

Hemagglutination tests indicated that the binding capacity of all lectin-TRITC conjugates, except LPA, was relatively strong. Specific hemagglutination activities of 1024, 512, 4096, 2048, and 16 units/mg lectin were determined for SBA, CON A, WGA, LOT, and LPA, respectively. Hemagglutination activity of all lectin-TRITC conjugates was completely inhibited in the presence of 0.1 M corresponding competitive sugar.

Viable, preinfective J2 were labeled with lectin-TRITC almost exclusively in the vicinity of the amphidial openings (Figs. 1-1a - 1-1c). Fluorescent lectin labeling often extended outward from these openings, suggesting that carbohydrates occur within amphidial secretions. Binding of

Fig. 1-1. Binding of fluorescent (rhodamine) lectin conjugates to Meloidogyne spp. second-stage juveniles (J2) and egg. a) Strong amphidial (SA) fluorescence of M. incognita race 3 (Mi3) labeled with Lotus tetragonolobus agglutinin. b) Binding of wheat germ agglutinin (WGA) to amphidial secretions (AS) of M. javanica (Mj) after α-galactosidase treatment. c) Weak amphidial fluorescence (WA) of M. incognita race 1 (Mi1) labeled with Limulus polyphemus agglutinin. d) Fluorescence of dead (D) vs. living (L) J2 of Mi1 after treatment with soybean agglutinin. e) Fluorescent cuticle (FC; note annulation) and amphidial ducts (AD) of Mj labeled with Concanavalin A after J2 exposure to α-galactosidase. f) Binding of WGA to egg shell (ES) of Mi3. (Note: Incandescent light provided to enhance J2 image in photographs a-d results in artifactual cuticular glow. True labeling of J2 cuticle by fluorescent lectin is presented in plate e.)


SA
10 µm
a
AS
10 µm
b
WA
10 µm
c
D
L
30 µm
d
AD
FC
10 µm
e
ES
10 µm
f

fluorescent lectins to any other portions of the nematode surface was rarely observed, except as indicated below for several glycohydrolase treatments. Nematodes that were straightened, vacuolated, and displayed no movement (nonviable) often exhibited strong labeling of the stylet, esophageal lumen, and especially the gut region after exposure to lectin-TRITC conjugates (Fig. 1-1d). No labeling of viable J2 with unconjugated TRITC was observed.

Few differences in fluorescent lectin labeling were observed among nonglycohydrolase-treated, preinfective J2 of the Meloidogyne spp. tested (Table 1-2). Amphids of Mi1, Mi3, and Mj labeled weakly with SBA, CON A, and LPA and strongly with WGA and LOT. No binding of TRITC-conjugated Limax flavus L. agglutinin (LFA; sialic-acid specific) to J2 was observed in preliminary tests (unpublished results), and binding of LPA-TRITC to J2 was not observed until combined with an improved fluorescent microscope light source (50 watt mercury lamp; Carl Zeiss, West Germany). Inhibition of lectin binding in the presence of the appropriate competitive sugar was only observed for LOT and LPA. All lectins tested bound to egg shells of Mi1, Mi3, and Mj (Fig. 1-1f) and binding was not inhibited in the presence of 0.1 M corresponding competitive sugar.

Differences in lectin labeling among the populations of Meloidogyne spp. tested were observed after preinfective

Table 1-2. Binding of fluorescent lectins to preinfective second-stage juveniles (J2) of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj).

	Mi1		Mi3		Mj	
Lectin	-Sug[a]	+Sug	-Sug	+Sug	-Sug	+Sug
SBA[b]	++[c]	++	+	+	++	++
WGA	+++	+++	+++	+++	++++	++++
CON A	++	++	++	++	++	++
LOT	+++	NF	+++	NF	++++	NF
LPA	++	NF	++	NF	++	NF

[a] J2 incubated in lectin solution +/- competitive sugar. Competitive sugars included: D-galactose for SBA; N-acetyl-glucosamine for WGA; D-mannose for CON A; L-fucose for LOT; N-acetyl-neuraminic acid for LPA.

[b] Soybean agglutinin (SBA), wheat germ agglutinin (WGA), Concanavalin A (CON A), Lotus tetragonobolus agglutinin (LOT), and Limulus polyphemus agglutinin (LPA).

[c] Epifluorescent microscope observations included: + = very weak amphidial fluorescence; ++ = weak aphidial fluorescence; +++ = strong amphidial fluorescence; ++++ = very strong amphidial fluorescence; NF = no fluorescence.

J2 were treated with various glycohydrolases (Table 1-3). Lectin labeling of J2 treated with glycohydrolases was compared to labeling of J2 which were incubated in enzyme buffer minus glycohydrolase. In most cases, enzyme activity was inhibited in the presence of the appropriate competitive sugar, except where indicated below.

Treatment of J2 with α-gal eliminated binding of SBA-TRITC to the amphids of Mj and Mi1, but not to amphids of Mi3. Binding of LOT-TRITC to the amphids of Mj was reduced by treatment of J2 with α-gal. The cuticle on the anterior half of the body of Mj and Mi1 labeled weakly with CON A-TRITC, with fluorescence of body annulation growing weaker from head to mid-body (Fig. 1-1e). Enzyme activity was not inhibited in the presence of 0.1M D-galactose for Mj. Similar cuticular labeling was not observed for J2 of Mi3 treated with α-gal, however, binding of CON A-TRITC to the amphids of Mi3 was eliminated. Binding of LPA-TRITC to the amphids of J2 was unchanged on Mj, increased on Mi1, and eliminated on Mi3 after treatment with α-gal.

Treatment of J2 of Mi1 with ß-glu reduced binding of WGA-TRITC to amphids and promoted binding of WGA-TRITC to the anterior cuticle of Mi1. Binding of LPA-TRITC to the amphids of J2 of Mj and Mi1 was eliminated by ß-glu treatment. However, binding of LPA-TRITC to the amphids of Mi3 increased after ß-glu treatment.

Table 1-3. Binding of fluorescent-lectins to preinfective second-stage juveniles (J2) of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) after J2 treatment with different glycohydrolases.

	Mi1		Mi3		Mj	
Enzyme (Lectin)	-Enz[a]	+Enz	-Enz	+Enz	-Enz	+Enz
α-galactosidase						
SBA[b]	++[c]	NF	+	+	++	NF
WGA	+++	+++	+++	+++	+++	+++
CON A	++	++,WC	++	NF	++	++,WC
LOT	+++	+++	+++	+++	+++	++
LPA	+	+++	++	NF	+	+
β-N-Acetyl-Glucosaminidase						
SBA	++	++	+	+	+	+
WGA	+++	++,WC	+++	+++	+++	+++
CON A	++	++	+	+	++	++
LOT	+++	+++	+++	+++	+++	+++
LPA	++	NF	NF	++	++	NF
α-mannosidase						
SBA	++	NF	++	++	++	++
WGA	+++	+++	+++	+++	+++	+++
CON A	++,WC	++,WC	++	NF	++	NF
LOT	+++	+++	++	++	+++	NF
LPA	++	NF	++	NF	++	NF

Table 1-3--continued.

Enzyme (Lectin)	Mi1 -Enz	Mi1 +Enz	Mi3 -Enz	Mi3 +Enz	Mj -Enz	Mj +Enz
α-L-fucosidase						
SBA	++	++	+++	+++	++	++
WGA	+++	+++	+++	+++	+++	+++
CON A	++,WC	++,WC	++	++	+++	+++
LOT	+++	++	+++	+++	+++	+
LPA	+++,WC	NF,WC	++	NF	++	NF
Neuraminidase						
SBA	NF	NF	+	+	+	+
WGA	+++	+++	+++	+++	+++	+++
CON A	++,WC	++,WC	+++	++	++,WC	+++,WC
LOT	+++	+++	+++	++	+++	+
LPA	++	NF	++	++	++	NF

[a] J2 incubated in lectin solution +/- prior treatment with select glycohydrolase.

[b] Soybean agglutinin (SBA), wheat germ agglutinin (WGA), Concanavalin A (CON A), Lotus tetragonobolus agglutinin (LOT), and Limulus polyphemus agglutinin (LPA).

[c] Epifluorescent microscope observations included: + = very weak amphidial fluorescence; ++ = weak aphidial fluorescence; +++ = strong amphidial fluorescence; ++++ = very strong amphidial fluorescence; NF = no fluorescence; WC = weak fluorescence of J2 cuticle along anterior half of body.

Binding of SBA-TRITC to the amphids of Mi1 and LPA-TRITC to the amphids of Mj, Mi1, and Mi3 was eliminated by α-man. Binding of LOT-TRITC to the amphids of Mj was eliminated by treatment of J2 with α-man, but α-man activity was not inhibited in the presence of 0.1 M mannose. Amphids of Mj and Mi3 did not label with CON A-TRITC after α-man treatment, but CON A-TRITC did bind to the anterior cuticle of Mi1 after treatment with α-man buffer plus or minus enzyme.

Binding of LOT-TRITC to the amphids of Mj and Mi1 was reduced and binding of LPA-TRITC to amphids of Mj, Mi1, and Mi3 was eliminated after treatment of J2 with α-fuc. Weak labeling of the anterior cuticle of Mi1 with CON A-TRITC and LPA-TRITC occurred after incubation of J2 in α-fuc buffer with or without the enzyme.

Treatment of J2 with neuraminidase partially inhibited binding of LOT-TRITC to amphids of Mj and Mi3, and completely inhibited binding of LPA-TRITC to amphids of Mj and Mi1. Neuraminidase treatment increased binding of CON A-TRITC to amphids of Mj and Mi1, but reduced binding of CON A-TRITC to amphids of Mi3. The anterior cuticle of Mj and Mi1 labeled weakly with CON A-TRITC after incubation in neuraminidase buffer with or without enzyme.

Discussion

Fluorescein isothiocyanate (FITC)-lectin conjugates were not used in fluorescence assays because untreated J2 of the Meloidogyne spp. populations examined strongly autofluoresced at the excitation wavelength of FITC. Difficulty with autofluorescence of C. elegans and P. redivivus at the excitation wavelength of FITC has also been reported (48). Lectins conjugated with rhodamine (TRITC) fluorophors were more appropriate for the study of lectin binding to nematodes. Preinfective J2 of Meloidogyne spp. were not visible when viewed through the TRITC microscope filter, except for body portions labeled with lectin-TRITC conjugates.

It is apparent from observations that nematode viability is critical for true labeling of nematodes with fluorescent lectins. Living (motile) J2 bound fluorescent lectin almost exclusively in the vicinity of the amphidial openings. The entire body of nematodes that were apparently dead fluoresced after TRITC-lectin treatment, especially in the gut region. The fluorescence of dead J2 was similar to the observations of enzymatically induced fluorescence of dead nematodes reported by Bird (11). This phenomenon may have influenced fluorescent observation of sialyl residues over the entire body of J2 of M. javanica as reported by Spiegel et al. (114). Labeling of sialyl residues with

fluorescent LPA was relatively weak and confined to the amphidial region of viable J2 of Meloidogyne spp. observed in these studies. Since the specific hemagglutination activity and absorbance ratio (550nm/280nm) of LPA-TRITC was relatively low compared to the other lectins tested, it may be possible that more sialic acid exists on the J2 surface than can be detected with fluorescent lectin probes. Lectin binding to the tail region of M. incognita has also been reported (77), but it was not observed in this study. The observed binding of fluorescent lectins to egg shells of Meloidogyne spp. in this investigation has been reported for eggs of M. javanica (113).

Only the binding of LOT and LPA to specific sugars on preinfective J2 were confirmed by competitive sugar inhibition, although all of the fluorescent lectins tested bound to amphids of preinfective J2 of the three Meloidogyne spp. populations examined. The binding of SBA, CON A, and WGA to J2 was apparently not specific for α-D-galactose, α-D-mannose, and ß-N-acetylglucosamine, respectively, since 0.1 M concentrations of these sugars were insufficient to inhibit binding of these TRITC-lectins to J2. Soybean agglutinin, CON A, and WGA bind to other molecular forms of galactose, mannose, and N-acetylglucosamine, respectively, and WGA has been reported to have multiple carbohydrate binding sites (38). It is possible that the affinity of

SBA, CON A, and WGA for carbohydrate-specific sites near amphidial openings was too strong to be inhibited by the competitive sugar solutions used in these assays. The binding of SBA, CON A, and WGA to amphidial secretions of J2 of Meloidogyne spp., however, may represent binding of these lectins to hydrophobic ligands (possibly lipids), as reported elsewhere (93). Incubation of J2 in fluorescent SBA, CON A, or WGA in the presence of 1,8 anilinonaphthalene sulfonic acid plus or minus competitive sugar may confirm the presence of hydrophobic binding since the hydrophobic and carbohydrate binding sites are independent of each other (93). The direct binding of unconjugated TRITC to surface lipids of J2 did not apparently occur in these assays since nematodes did not fluoresce after incubation in unconjugated TRITC.

The greater intensity of fluorescent labeling by LOT and WGA conjugates may be due to their relatively higher binding capacities. Lack of differential lectin labeling among nonglycohydrolase-treated, preinfective J2 of Mi1, Mi3, and Mj makes it difficult to extrapolate a potential role of surface carbohydrates in the specificity of pathogenicity (58). Substances to which lectins were bound were concentrated and sometimes emanated from the amphidial region of J2 of Meloidogyne spp., and this has also been reported for invasive juveniles of pathotypes of potato cyst

nematode and other populations of Meloidogyne spp. (36, 76). Since this is the portion of the nematode body around which some postinfectional, incompatible plant responses occur (43, 61), it may be possible that carbohydrates in amphidial secretions of postinfectional J2 affect plant-nematode interactions.

Results of experiments involving glycohydrolases suggested that carbohydrates located in the amphidial region of J2 occurred in complexes and that these complexes were structurally different among populations of Meloidogyne spp. The inability of enzymes to alter carbohydrate residues on some nematode surfaces may be a reflection of the substrate specificity exhibited by glycohydrolases (2, 125, 132). Enzyme treatment did reveal cuticular carbohydrates, especially mannose and/or glucose, on the anterior half of some J2 , and sialyl residues were often removed from J2 amphids by a number of different glycohydrolases. This may indicate that sialic acids are some of the outermost residues present in the carbohydrate complexes which apparently exist in amphidial secretions of J2 of Meloidogyne spp. Whether surface carbohydrate changes similar to those reported for glycohydrolase treatments occur once Meloidogyne spp. J2 enter plant roots is unknown. Alteration of the surface carbohydrates of J2 of Meloidogyne

spp., and subsequent plant root tissue responses to infection by treated J2, may provide insight into the specificity of plant-nematode interactions.

CHAPTER 3
ROOT TISSUE RESPONSE OF TWO RELATED SOYBEAN CULTIVARS TO INFECTION BY LECTIN-TREATED *MELOIDOGYNE* SPP.

Introduction

Plant incompatibility with nematodes often results from active plant defense reactions to infection by phytoparasitic nematodes (37, 39, 42, 58, 59, 97, 127). Although several mechanisms of active incompatibility have been proposed, little is known about nematode characteristics that may elicit plant responses that are incompatible with nematode development and their relation to specifity in plant-nematode interactions (58). The occurrence of physiological races of phytoparasitic nematodes (108, 119) suggests that specific interactions occur between nematodes and plant genotypes, and that populations of phytoparasitic nematodes adapt to overcome incompatibility.

Evidence that surface biochemistry, especially glycoconjugates of cells and organisms, promotes specificity in plant-microbe interactions has been the subject of several reviews (3, 21, 23, 62, 106). Keen (62) suggested that biochemical surface interactions were important in the specificity of incompatibility in gene-for-gene systems

between plant cultivars and microbial pathogens. It is unclear if surface interactions are important in plant-nematode incompatibility; however, the existence of carbohydrates on the surface of nematodes, and evidence that surface carbohydrates may be important in interactions between nematodes and microbes has been reported and summarized (47, 135, 136). Zuckerman and Jansson (136) proposed that interaction between nematodes and other organisms may be altered by obliteration or blocking of carbohydrates on the nematode surface.

To evaluate this concept with respect to specificity in plant-nematode interactions a model system was chosen which consisted of two related soybean cultivars, 'Centennial' and 'Pickett 71', and three Meloidogyne spp. populations that differed in compatibility with Centennial soybean. The incompatibility of Centennial soybean with Meloidogyne incognita has been associated with a hypersensitive reaction (HR) of soybean root tissue in the region of invading second-stage juveniles (J2) (61). The HR was strongly correlated with the accumulation of glyceollin, but biotic elicitors of the HR were not identified (60).

The surface carbohydrates of several populations of cyst and root-knot nematodes, including J2 of Florida populations of M. incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) (see Chapter 2 above), have been characterized

with fluorescent lectin probes (36, 76, 95). Lectins bound to infective J2 of Meloidogyne spp. primarily in the region proximate to cephalic chemosensillae (see Chapter 2 above, 77). In the study reported here, surface carbohydrates of J2 of Mi1, Mi3, and Mj were "blocked" with lectins and subsequent soybean root tissue responses to lectin-treated J2 were observed.

Materials and Methods

Populations of Meloidogyne incognita races 1 and 3 and M. javanica (Treub) Chitwood were maintained in greenhouse culture on roots of 'Rutgers' tomato (Lycopersicon esculentum Mill.) and 'Black Beauty' eggplant (Solanum melongena L.). Meloidogyne spp. populations were typified by adult female perineal patterns, second-stage juvenile (J2) lengths, and performance on differential hosts (101). Species identifications were also confirmed by three independent nematode taxonomists (A. M. Golden, Beltsvilee, MD; J. G. Baldwin, Riverside, CA; J. D. Eisenback, Blacksburg, VA). Eggs of each nematode population were extracted from host roots with 0.53% NaOCl for 30 seconds (46) and hatched at room temperature on a Baermann funnel. Preinfective J2 which had hatched within 48 hours were used as test organisms in each experiment.

Surface carbohydrates of Meloidogyne spp. J2 were blocked by incubating nematodes in solutions containing unconjugated, purified soybean agglutinin (SBA), wheat germ agglutinin (WGA), Lotus tetragonolobus agglutinin (LOT), Concanavalin A (CON A), or Limax flavus agglutinin (LFA) (E-Y Labs, San Mateo, CA). The sugar specificity, appropriate lectin buffers, corresponding competitive sugars, and procedure used to determine the specific hemagglutination activity for each lectin were described above in Chapter 2. The sugar specificity of LFA is N-acetyl-neuraminic (sialic) acid, and LFA assays were conducted in buffer which contained 0.05 M Tris-saline plus 0.01 M $CaCl_2$ at pH 7.5.

Preinfective J2 of Mi1, Mi3, and Mj were concentrated in the appropriate buffer or in distilled water by centrifugation at 1000g for 3 minutes. Treatments for each lectin included incubating J2 (approx. 2000 J2) of each population in solutions of lectin (200 µg/ml), lectin (200 µg/ml) plus 0.1M competitive sugar, and 0.1M sugar minus lectin for 2 hours at 4°C. Juveniles in these solutions were used as direct (unwashed treatment) inoculum for subsequent soybean root challenge. Since a 0.1 M solution of sialic acid in LFA buffer was quite acidic (pH ~ 3.0), a soybean root penetration bioassay was conducted to address the effect of sialic acid and acidity on the activity of J2

of Meloidogyne spp. (see Appendix D below). In addition, J2 exposed to each lectin and sugar treatment were washed three times in buffer and subsequently used as inoculum for soybean root challenge. Control treatments included J2 in buffer and J2 in distilled water.

Two related cultivars of soybean (Glycine max (L.) Merr.), 'Pickett 71' and 'Centennial', were used for root tissue challenge by treated J2 of Meloidogyne spp. It has been reported that Pickett 71 was compatible and Centennial was incompatible with M. incognita, and both soybean cultivars were compatible with M. javanica (61). Seeds of each variety were dusted with Thiram 75WP (Kerr-McGee Chemical Corp., Jacksonville, FL) and germinated in moist germination paper which was rolled up (ragdolls) and incubated in the dark at 27°C. Newly germinated soybeans with roots 3-5 cm long were placed on trays containing autoclaved Astatula fine sand (hyperthermic, uncoated typic quartzipsamments) and the root tips covered with a small amount of sand. Nematode suspensions (approx. 600 J2) from each treatment were placed on separate soybean root tips (61, 75). Trays containing inoculated soybeans in sand were incubated in the dark at 27°C. Treatments were arranged as a 3x5x8x2 factorial including three Meloidogyne spp. populations, five lectins, eight treatments, and two soybean

varieties. There were seven replicates of each treatment combination.

Soybeans were removed from trays and their roots washed free of sand and any nematodes that had not penetrated approximately 40 hours after inoculation. The seedlings were then placed on moist germination paper and the inoculated portions of the roots were marked on the paper. The germination paper which contained inoculated seedlings was covered with an additional piece of moist germination paper, carefully rolled into ragdolls, and incubated in the dark at 27°C. This "pulse inoculation" was used to ensure that nematodes observed within roots had entered within 40 hours of inoculation.

Inoculated soybean root segments were excised and immediately fixed in 10% alcoholic formalin (1:9, formalin:95% EtOH, v/v) a total of 5 days after inoculation. Additional treatments of J2 of Mi3 in water applied to Centennial and Pickett 71 soybean roots were excised 20 days after inoculation and processed according to these protocols. Fixed root segments were dehydrated through a tert-butyl alcohol series and embedded in paraffin. Serial sections (12 µm) were mounted on glass slides, stained with safranin-fast green, and observed under light microscopy. Sections from seven replicates of each treatment combination were observed and the most frequent tissue responses to

nematode infection were determined. This experiment was repeated once.

Results

Hemagglutination assays indicated that the binding capacity of pure lectins was relatively strong, except for LFA. Specific hemagglutination activities of 4096, 4096, 8192, 8192, and 4 units/mg lectin were determined for SBA, CON A, WGA, LOT, and LFA, respectively. Hemagglutination activity of all lectins was completely inhibited in the presence of 0.1 M corresponding competitive sugar.

The most common or primary responses of soybean root tissue to infection by Meloidogyne spp. J2 are reported in Tables 2-1 to 2-5. Infective juveniles incubated in buffer or water became enlarged and induced giant cell formation in compatible interactions but remained vermiform and were associated with a hypersensitive reaction in incompatible combinations (Fig. 2-1). Giant cells of normal appearance were usually observed in roots of Pickett 71 and Centennial soybean 5 and 20 days after roots were exposed to Mi3 incubated in buffer or water. Giant cells associated with Mi3 controls in Centennial soybean sometimes contained granular cytoplasm. No gall formation, evidence of hyperplasia of pericycle cells adjacent to giant cells, or development of Mi3 past third-stage juvenile was observed in

Table 2-1. Primary tissue reactions of 'Pickett 71' (P) and 'Centennial' (C) soybean roots 5 days after their exposure to second-stage juveniles of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) that were incubated in soybean agglutinin (SBA) solution (200 µg/ml) and (or) 0.1M galactose (gal) solution prior to inoculation.

	Mi1		Mi3		Mj	
Treatment	P	C	P	C	P	C
Washed[a]						
SBA	GC, GRCY[b]	HR, NR	GC, NR	HR, EGC	GC, S	GC, S
SBA + gal	GC	HR	GC, GRCY	HR, NR	GC, GRCY	GC, S
gal	GC, GRCY	HR	GC, GRCY	HR, NR	GC, GRCY	GC
Unwashed						
SBA	GC, GRCY	HR, EGC	GC, S	HR, EGC	GC, S	GC, S
SBA + gal	GC, GRCY	HR, EGC	EGC, GRCY	HR, EGC	GC, EGC	GC, S
gal	GC, S	HR, GRCY	GC, S	HR, NR	GC, S	GC, S
Buffer	GC, GRCY	HR, GRCY	GC, S	GC, GRCY	GC, S	GC, S
Distilled H_2O	GC, S	HR	GC, S	GC, GRCY	GC, S	GC, S

[a] Second-stage juveniles were incubated in treatment solution and washed three times with buffer before inoculation.

[b] GC= giant cells; GRCY= granular cytoplasm within giant cells; HR= hypersensitive response; NR= no response; EGC= early giant cells; S= swollen juvenile.

Table 2-2. Primary tissue responses of 'Pickett 71' (P) and 'Centennial' (C) soybean roots 5 days after their exposure to second-stage juveniles of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) that were incubated in Concanavalin A (CON A) solution (200 µg/ml) and (or) 0.1M mannose (man) solution prior to inoculation.

	Mi1		Mi3		Mj	
Treatment	P	C	P	C	P	C
Washed[a]						
CON A	GC, NR[b]	NR, HR	GC, S	HR	GC	GC, S
CON A + man	GC, GRCY	HR, NR	GC, S	HR, NR	GC, S	GC, S
man	GC, S	HR	GC	NR, GC	GC, GRCY	GC, GRCY
Unwashed						
CON A	GC, S	HR	GC, EGC	HR, NR	GC, S	GC, S
CON A + man	GC, S	HR	GC, S	HR, EGC	GC, S	GC, S
man	GC, S	NR, EGC	GC, S	GC, GRCY	GC, S	GC, S
Buffer	GC, S	HR, NR	GC, S	GC, GRCY	GC, S	GC, S
Distilled H_2O	GC, S	HR, GRCY	GC, S	GC, EGC	GC, S	GC, S

[a] Second-stage juveniles were incubated in treatment solution and washed three times with buffer before inoculation.

[b] GC= giant cells; GRCY= granular cytoplasm within giant cells; HR= hypersensitive response; NR= no response; EGC= early giant cells; S= swollen juvenile.

Table 2-3. Primary tissue responses of 'Pickett 71' (P) and 'Centennial' (C) soybean roots 5 days after their exposure to second-stage juveniles of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) that were incubated in Lotus tetragonolobus agglutinin (LOT) solution (200 µg/ml) and (or) 0.1M fucose (fuc) solution prior to inoculation.

	Mi1		Mi3		Mj	
Treatment	P	C	P	C	P	C
Washed[a]						
LOT	GC, S[b]	HR, EGC	GC, S	HR, EGC	GC, S	GC, GRCY
LOT + fuc	GC, S	HR, NR	GC, NR	HR, GRCY	GC, S	GC, S
fuc	GC, GRCY	HR	GC, EGC	HR, NR	GC, S	GC, S
Unwashed						
LOT	GC, GRCY	HR	GC, GRCY	HR, EGC	GC, S	GC, GRCY
LOT + fuc	GC, GRCY	HR, GRCY	GC, S	HR, EGC	GC, EGC	GC, NR
fuc	GC, GRCY	HR, GRCY	GC, S	HR, EGC	GC, S	GC, S
Buffer	GC, S	HR	GC, S	GC, EGC	GC, S	GC, S
Distilled H_2O	GC, S	HR	GC, S	GC, S	GC, S	GC, S

[a] Second-stage juveniles were incubated in treatment solution and washed three times with buffer before inoculation.

[b] GC= giant cells; GRCY= granular cytoplasm within giant cells; HR= hypersensitive response; NR= no response; EGC= early giant cells; S= swollen juvenile.

Table 2-4. Primary tissue responses of 'Pickett 71' (P) and 'Centennial' (C) soybean roots 5 days after their exposure to second-stage juveniles of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) that were incubated in wheat germ agglutinin (WGA) solution (200 µg/ml) and (or) 0.1M N-acetylglucosamine (NAcGlu) solution prior to inoculation.

	Mi1		Mi3		Mj	
Treatment	P	C	P	C	P	C
Washed[a]						
WGA	GC, EGC[b]	HR, NR	GC, NR	HR, NR	GC, NR	GC
WGA + NAcGlu	GC, NR	HR, NR	GC, EGC	HR, NR	GC, S	GC, S
NAcGlu	GC, HR	HR, GRCY	GC, S	NR, HR	GC, S	GC, S
Unwashed						
WGA	GC, NR	HR, NR	GC, S	HR, GRCY	GC, S	GC, GRCY
WGA + NAcGlu	GC, HR	NR, HR	GC, S	HR	GC, S	GC, S
NAcGlu	GC, HR	HR	GC, S	HR, EGC	GC, S	GC, S
Buffer	GC, S	HR, NR	GC, S	GC, NR	GC, S	GC, S
Distilled H_2O	GC, S	HR, NR	GC, S	GC, GRCY	GC, S	GC, S

[a] Second-stage juveniles were incubated in treatment solution and washed three times with buffer before inoculation.

[b] GC= giant cells; GRCY= granular cytoplasm within giant cells; HR= hypersensitive response; NR= no response; EGC= early giant cells; S= swollen juvenile.

Table 2-5. Primary tissue responses of 'Pickett 71' (P) and 'Centennial' (C) soybean roots 5 days after their exposure to second-stage juveniles of Meloidogyne incognita races 1 and 3 (Mi1, Mi3), and M. javanica (Mj) that were incubated in Limax flavus agglutinin (LFA) solution (200 µg/ml) and (or) 0.1M N-Acetylneuraminic (sialic) acid solution prior to inoculation.

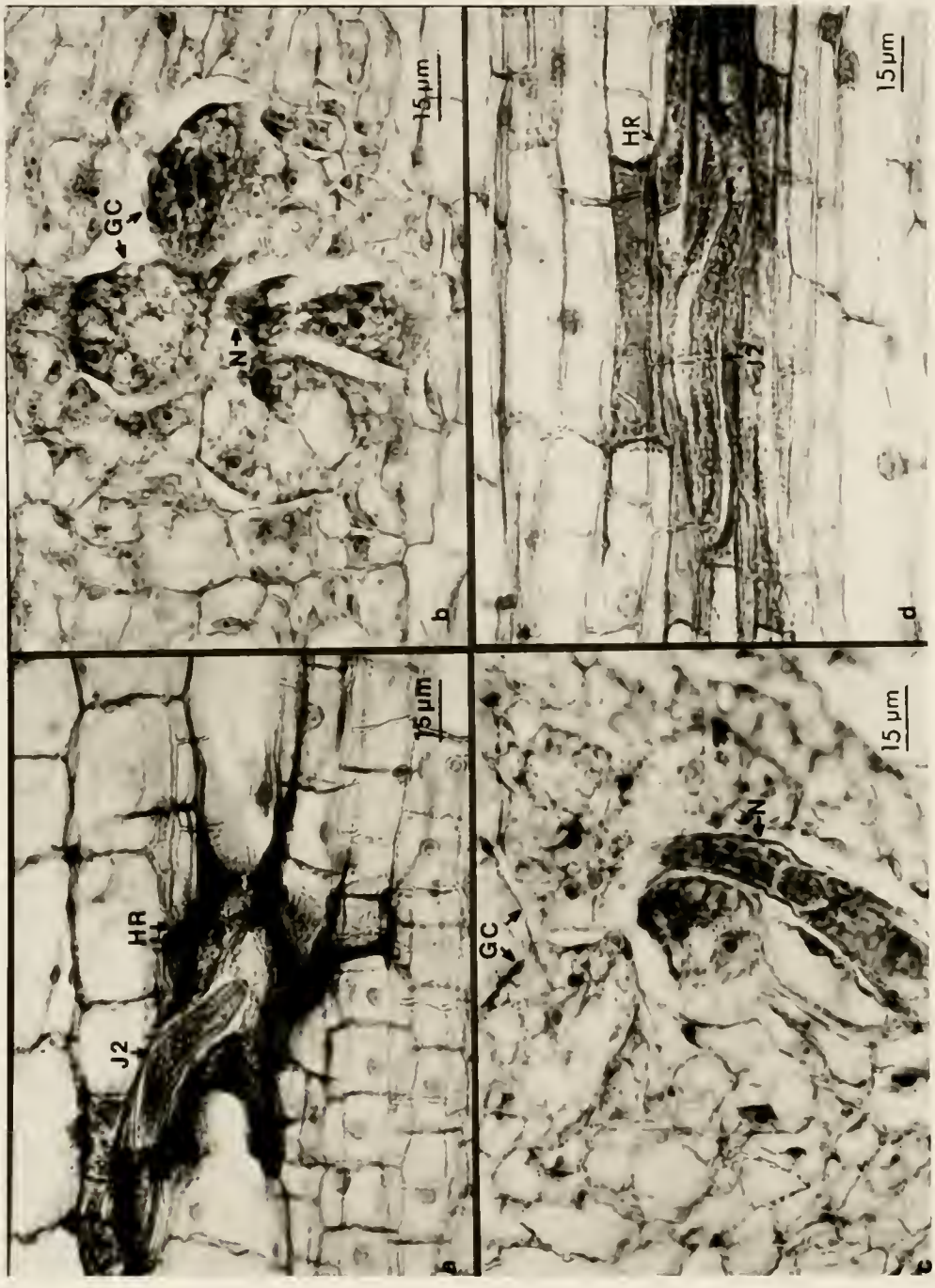
	Mi1		Mi3		Mj	
Treatment	P	C	P	C	P	C
Washed[a]						
LFA	GC, S[b]	NR, HR	GC, EGC	HR, NR	GC, S	GC, S
LFA + sialic	GC, S	HR	NR, EGC	HR, EGC	GC, S	GC, S
sialic	GC, GRCY	HR, GRCY	GC, EGC	HR	GC, S	GC, S
Unwashed						
LFA	--[c]	--	--	--	--	--
LFA + sialic	--	--	--	--	--	--
sialic	--	--	--	--	--	--
Buffer	GC, S	HR, GRCY	GC, S	GC, EGC	GC, S	GC, S
Distilled H_2O	GC, S	HR, NR	GC, S	GC, GRCY	GC, S	GC, S

[a] Second-stage juveniles were incubated in treatment solution and washed three times with buffer before inoculation.

[b] GC= giant cells; GRCY= granular cytoplasm within giant cells; HR= hypersensitive response; NR= no response; EGC= early giant cells; S= swollen juvenile.

[c] Few or no nematodes observed within soybean root tissue.

Fig. 2-1. Response of 'Centennial' soybean root tissue within 5 days of infection by lectin-treated second-stage juveniles (J2) of Meloidogyne spp. a) Hypersensitive reaction (HR) to M. incognita race 1 treated with soybean agglutinin. b) Giant cells (GC) induced by the untreated nematode (N), M. incognita race 3. c) Giant cells induced by M. javanica treated with soybean agglutinin plus galactose. d) Hypersensitive reaction induced by M. incognita race 3 treated with N-acetylglucosamine.


J2
HR
15 µm
a
GC
N
15 µm
b
GC
N
15 µm
c
HR
J2
15 µm
d

Centennial root tissue 20 days after inoculation with J2 of Mi3.

The primary tissue response of both soybean cultivars to J2 of Mi1 or Mj exposed to any lectin or sugar treatment was essentially unchanged from that of tissues infected by J2 incubated in buffer or water. Lectin or sugar treatment of J2 of Mi3 did not influence host response in Pickett 71, but in Centennial soybean roots, treatment of J2 of Mi3 with any lectin or sugar stimulated a hypersensitive response rather than giant cells.

Soybean tissue responses to infection by J2 that were atypical of the primary tissue response sometimes occurred frequently enough to warrant report. The occurrence of early giant cells in Centennial tissue challenged by Mi1 treated with SBA and SBA plus galactose, unwashed (Table 2-1), was in contrast to the primary response of hypersensitivity. Early giant cell formation was also observed in Centennial challenged by Mi1 treated with mannose, unwashed (Table 2-2), and Mi1 treated with LOT, washed (Table 2-3).

Second-stage juveniles of Meloidogyne spp. were observed in soybean roots with no apparent plant tissue reaction. This was the primary observation in Centennial root tissue with Mi1 treated with CON A, washed; Mi1 treated with mannose, unwashed; Mi3 treated with mannose, washed;

Mi1 treated with WGA plus N-Acetyl-D-glucosamine (NAcGlu), unwashed; Mi3 treated with NAcGlu, washed; and Mi1 treated with LFA, washed (Tables 2-2, 2-4, 2-5). No tissue response was observed in Pickett 71 to Mi3 treated with LFA plus sialic acid, washed (Table 2-5). A hypersensitive response was sometimes observed in Pickett 71 challenged by Mi1 treated with NAcGlu, washed; Mi1 treated with WGA plus NAcGlu, unwashed; and Mi1 treated with NAcGlu, unwashed. Almost no J2 were observed in soybean roots challenged by any population of Meloidogyne spp. that received unwashed LFA and sialic acid treatments. When J2 of these treatments were occasionally observed in soybean roots, tissue response was similar to comparable treatments with SBA.

Discussion

It appears that incubation of J2 of Mi1 in the lectins or sugars tested had little effect on soybean root tissue response in the incompatible pathosystem. If the concept that preformed sites (carbohydrate moieties) on the surface of Mi1 J2 are responsible for recognition by plant cell surface receptors and subsequent plant defense reaction were valid, blockage of these sites with lectins should have prevented the HR observed in Centennial root tissue. In a few instances, early giant cell formation in Centennial by Mi1 was noted, but the HR was much more common. The more

frequent occurrence of HR in Pickett 71 exposed to Mi1 treated with N-acetyl-D-glucosamine may indicate an alteration of nematode surface carbohydrates which promoted incompatibility.

The fate of lectin bound to J2 of Meloidogyne spp. once the nematode entered soybean root tissue is questionable. Unwashed treatments were included in this study to ensure that J2 were present in an environment of lectin and (or) sugar until they penetrated roots. No treatments, however, had an effect on the compatible interaction between Mj and root tissue of either soybean cultivar. Differences in the quantity, balance, or accessibility of secretory carbohydrates to potential plant receptors, however, may promote incompatibility or compatibility. Previous reports indicated that lectins bound to amphidial secretions of J2 of Meloidogyne spp. (see Chapter 2 above, 77), but the rates of production of amphidial secretions by J2 of Meloidogyne spp. and the quantities of this material that are sloughed off in plant tissue or the soil environment are unknown.

The occurrence of normal giant cells in Centennial 20 days after inoculation of root tips with J2 of Mi3 incubated in water was unexpected. This host-parasite relationship was apparently incomplete compared to Mi3 in Pickett 71, however, since pericyclic hyperplasia and

nematode development were strongly inhibited 20 days after inoculation. Differences in the degree of incompatibility have been reported for several soybean cultivars and M. incognita populations (105). Treatment of J2 of Mi3 with any lectin or sugar promoted active incompatibility (HR) in Centennial soybean, and may actually have facilitated recognition of invasive Mi3 and subsequent defense response by the plant. The lack of specificity of lectin or sugar effects in the Mi3-Centennial interaction makes it seem unlikely that alteration of surface carbohydrate composition of preinfective J2 was responsible for promoting incompatibility. If one considers the interaction of nematode surface carbohydrates with potential plant cell surface receptors as a "lock and key" phenomenon, however, it may be feasible that a slight alteration in surface carbohydrate compostion was sufficient to promote incompatibility to Mi3 in Centennial soybean roots. Possibly a greater alteration of the carbohydrates examined here on Mi1 and Mj, or alteration of surface carbohydrates not examined in these studies, would influence their host-parasite interactions.

Treatment of J2 of Mi3 with lectin or sugar may have stimulated the production of a substance by the nematode that induces HR in Centennial soybean roots. Juveniles of Mi1 may inherently have the capacity to induce HR while J2

of Mj cannot promote incompatibility in soybean no matter what the treatment. For some populations, such as in the case of Mi3, incompatibility may be a process that can be stimulated. Conversely, substances produced by J2 of Meloidogyne spp. (ie. amphidial or stylet secretions) may be essential to induce compatibility between host and parasite (82), and these substances were altered sufficiently in Mi3 to inhibit compatibility in Centennial soybean roots.

The ability of J2 of Mi1, Mi3, and Mj to penetrate roots of both soybean varieties was apparently strongly impaired when J2 were introduced to roots in a solution that contained LFA, sialic acids, or combination of the two. It was not determined if J2 penetrated and exited from roots within the 40 hour "pulse inoculation." Inhibition of soybean root penetration by J2 of Meloidogyne spp. treated with sialic acid occurred in similar tests (see Appendix D below), and this appeared to be more than an effect of the low pH of a 0.1 M sialic acid solution. It has been reported that a single soil application of CON A, and LFA at relatively higher concentrations, significantly reduced galling of tomato roots induced by M. incognita (74), but contact and effect of active lectins to nematodes in this system was unclear. Little effect of CON A on soybean root penetration by J2 of any Meloidogyne spp. population was observed in this investigation, even though J2 were

incubated in CON A solution (200 µg/ml) before their application to roots in soil. Sialic acids have been reported to be important in the adhesion of conidia of Meria coniospora to nematode surfaces, especially at the chemosensory organs (50-52). In the studies reported herein, nematode viability after LFA and sialic acid treatment was confirmed by infectivity of washed J2, and also viable J2 of Meloidogyne spp. have been observed microscopically after similar treatment (see Chapter 2 above). Removal of sialic acids from amphidial secretions by a number of selective glycohydrolases (see Chapter 2 above) suggests that sialic acids are some of the outermost carbohydrate residues of amphidial glycoconjugates. Whether sialic acid residues proximate to nematode chemosensillae have a masking or regulatory effect similar to those observed in other animal systems (104) should be the subject of further investigation.

CHAPTER 4
QUANTIFICATION OF LECTIN BINDING TO SECOND-STAGE JUVENILES OF *MELOIDOGYNE* SPP.

Introduction

Lectins, proteins that bind to specific carbohydrate residues, are excellent probes for the study of the carbohydrate chemistry of biological surfaces. Lectins, and their involvement in recognition and specificity in plant-microbe interactions, have been the subject of several reviews (3, 21, 23, 31, 70, 106, 107). The surface carbohydrate chemistry of nematodes of various parasitic habits has been characterized by several techniques, including lectin probes. Some of this work has been summarized by Zuckerman and Jansson (136), who postulated that blockage or obliteration of specific carbohydrates on nematode surfaces may alter interactions between nematodes and other organisms. Examples include the association of surface carbohydrates in antigenicity and chemoresponse of nematodes parasitic to animals (14, 72, 85) and the interaction of nematodes with nematophagous fungi (17, 47, 80).

The potential involvement of nematode surface carbohydrates in recognition and specificity in plant-

nematode interactions, however, has not been clearly demonstrated (47, 58, 135, 136). Application of fluorescent-lectin probes to differentiate nematode species and pathotypes on the basis of their surface carbohydrates has been attempted (see Chapter 2 above, 36, 76, 95). However, the intensity of fluorescence of rhodamine-labeled lectins that bound to cephalic sensory structures of Meloidogyne (Goeldi, 1887) spp. second-stage juveniles (J2) differed among the lectins tested (see Chapter 2 above, 76). Quantification of lectin binding to nematode pathotypes may reveal differences in relative carbohydrate content that were not detectable in fluorescent-lectin assays. Lectins labeled with hemocyanin and with tritium have been used to quantify relative amounts of carbohydrates on the surface of nematodes and bacteria, respectively (77, 82). The difficulty in production and handling of radiolabeled lectins, and the sophisticated equipment required to observe hemocyanin conjugates on the nematode surface, limits the practicality of these methods. We have developed a microfiltration enzyme-linked lectin assay (27, 78, 84, 120) to quantify the amount of lectin that binds to J2 of Meloidogyne spp.

Materials and Methods

Populations of *Meloidogyne incognita* (Kofoid & White) Chitwood race 3 (Mi3), *M.incognita* race 1 (Mi1), and *Meloidogyne javanica* (Treub) Chitwood (Mj) were maintained in greenhouse culture on roots of 'Rutgers' tomato (*Lycopersicon esculentum* Mill.) and 'Black Beauty' eggplant (*Solanum melongena* L.). *Meloidogyne* spp. populations were identified by adult female perineal pattern, second-stage juvenile (J2) length, and development on differential hosts (101). Species identifications were also confirmed by three independent nematode taxonomists (A. M. Golden, Beltsville, MD; J. G. Baldwin, Riverside, CA; J. D. Eisenback, Blacksburg, VA). Eggs of each nematode population were extracted from host roots in 0.53% NaOCl solution for 30 seconds (46) and hatched at room temperature on a Baermann pan. Preinfective J2 which had hatched within 48 hours were used as test organisms in each experiment.

The quantity of lectin binding to preinfective J2 of Mi1, Mi3, and Mj was determined using a microfiltration enzyme-linked lectin assay (78, 84). Lectins conjugated with horseradish peroxidase (HRP) were purchased from E-Y Labs (San Mateo, CA) and included soybean agglutinin (SBA), wheat germ agglutinin (WGA), *Lotus tetragonolobus* agglutinin (LOT), Concanavalin A (CONA), and *Limulus polyphemus* agglutinin (LPA). The sugar specificity, corresponding

buffer solutions, and procedure for determination of the specific hemagglutination activity of each lectin were presented in Chapter 2.

Ninety six-well microfilter plates (SV-96, Millipore Corp., Bedford, MA) with a 5 µm pore size were incubated with 200 µl of 1.0% bovine serum albumin (BSA) in phosphate buffer saline (PBS), pH 7.2, at 37°C for 2 hours. These plates were washed three times with PBS on a microfiltration apparatus (Millipore Corp., Bedford, MA) prior to their use in the following assay (Fig. 3-1).

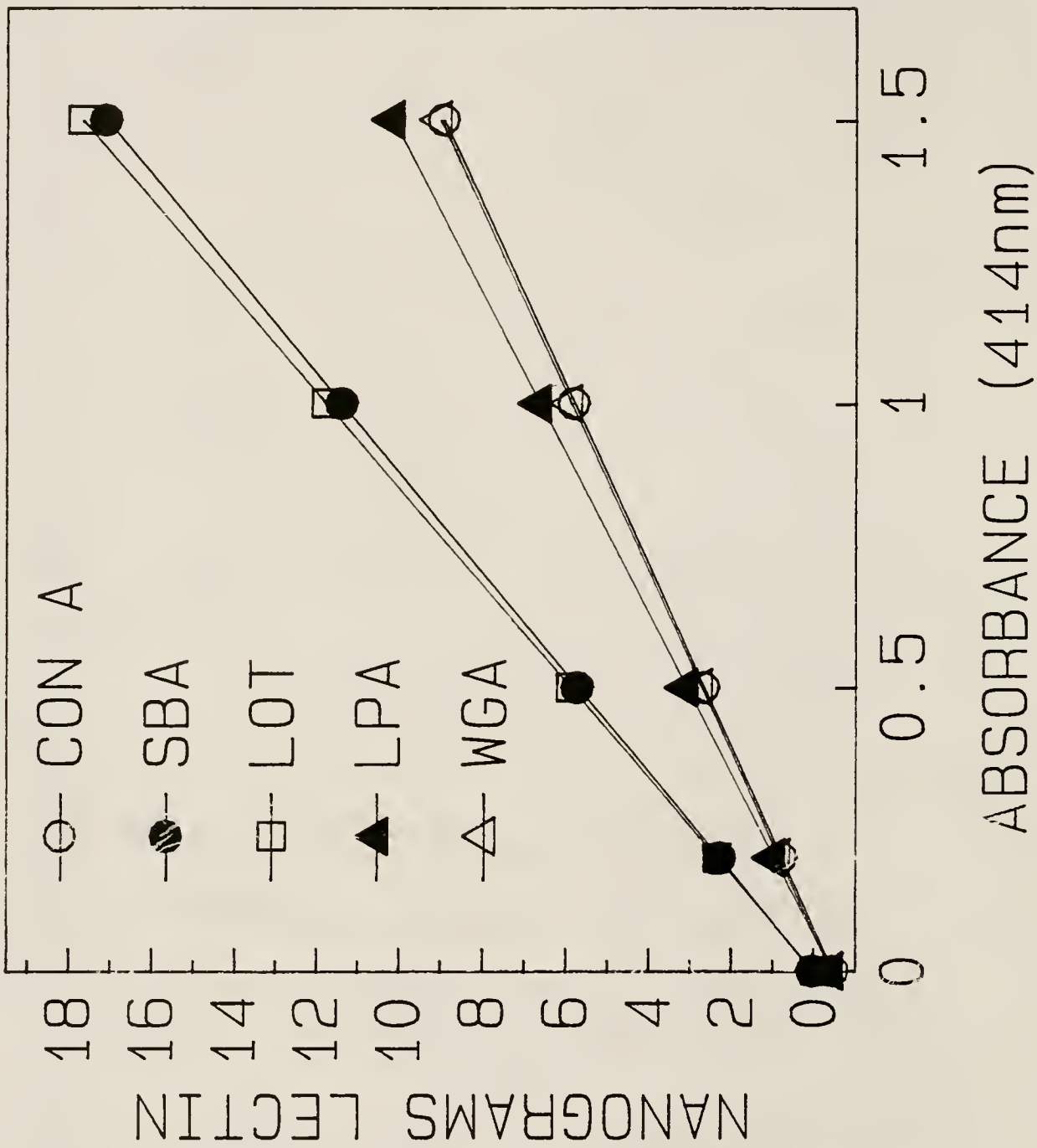
Preinfective J2 of each Meloidogyne spp. population were concentrated into the appropriate buffer for each lectin-HRP conjugate. Nematodes were incubated in 500 µl of lectin-HRP solution (200 µg/ml) for 2 hours at 4°C. Control treatments included J2 in buffer (untreated J2) and 500 µl lectin solution (200µg/ml) minus J2 (lectin wash). Competitive sugar controls similar to ones described in Chapter 2 were omitted due to insufficient numbers of freshly-hatched Meloidogyne spp. J2. Five, 100-µl samples from each treatment combination (approx. 2000 J2 suspended in lectin solution) were placed in separate wells on a microfilter plate. Each well was washed five times with the appropriate lectin buffer on a microfiltration apparatus. The treated J2 or lectin wash in each well were suspended in 100 µl of buffer and transferred to separate microcentrifuge

Figure 3-1. Microfiltration plates and suction apparatus (Millipore Corp., Bedford, MA).

tubes. The volume of each microcentrifuge tube was increased to 250 µl with buffer, and 50 µl of suspension were withdrawn from each tube to quantify the number of J2 per 50-µl sample. Four, 50-µl suspensions of treated J2 or lectin wash were transferred from each tube to separate wells on a fresh 96-well microfilter plate. One hundred microliters of peroxidase substrate,([2, 2'-azino-di-(3-ethyl-benzthiazoline) sulfonic acid]) (ABTS, Sigma Chemical Corp., St. Louis, MO) were added to each of these wells and allowed to incubate at room temperature in the dark for 30 minutes (110). The solution from each well was transferred to corresponding wells on a 96-well enzyme immunoassay plate using a microfiltration apparatus to remove J2. Absorbance (414 nm) of solution in each well was determined on an automated microplate reader (Model EL309, Bio-Tek Instruments, Winooski, VT). Twenty separate absorbance values were determined for each treatment combination.

The absorbance values determined in this assay were compared to the linear portion of a standard curve prepared for each lectin-HRP conjugate (Fig. 3-2). Standard curves were prepared by diluting (1:1, v/v) 50-µl volumes of lectin-HRP solution across a 96-well EIA plate and adding 100 µl of ABTS solution per well. The quantity of lectin which adsorbed to a single J2 was determined by dividing the

Figure 3-2. Standard curves of lectin-peroxidase conjugates. Concanavalin A (CON A) = 6258.651x-468.338, R^2 = 0.957; Soybean agglutinin (SBA) = 11,388.817x+12.636, R^2 = 0.995; *Lotus tetragonobolus* agglutinin (LOT) = 11,791.765x-31.613, R^2 = 0.995; *Limulus polyphemus* agglutinin (LPA) = 7069.594x-459.472, R^2 = 0.977; Wheat germ agglutinin (WGA) = 6282.532x-428.531, R^2= 0.967.


CON A
SBA
LOT
LPA
WGA
NANOGRAMS LECTIN
0
2
4
6
8
10
12
14
16
18
0
0.5
1
1.5
ABSORBANCE (414nm)

observed lectin value by the number of J2 estimated for that sample (approx. 500-2000 J2). The test was repeated once.

Results

Hemagglutination tests indicated that the binding capacity of all HRP-lectins, except LPA, was relatively strong. Specific hemagglutination activities of 512, 512, 4096, 2048, and 8 units/mg lectin were determined for SBA, CON A, WGA, LOT, and LPA, respectively. Hemagglutination activity of all lectin-HRP conjugates was completely inhibited in the presence of 0.1 M corresponding competitive sugar.

It was critical to incubate the first plate in BSA and transfer the treated J2 to a clean microfilter plate before addition of peroxidase substrate to reduce background to negligible levels. The reduction in background levels allowed the detection of differential amounts of lectin in microplate wells which contained lectin-treated nematodes. No peroxidase activity above background levels was detected among untreated, preinfective *Meloidogyne* spp. J2 and lectin wash treatments.

Relatively high numbers of J2 per sample (approx. 2000 J2 per microplate well) were required at the initiation of each experiment. As many as 75% of the J2/well remained in the first microfilter plate after transfer of washed

nematodes to a fresh microfilter plate for peroxidase substrate reaction. No J2 were observed in the wash solution which had passed through the first microfilter plate. Thus, estimation of the number of nematodes in a sample was made upon transfer of washed nematodes to the second microfilter plate.

Approximately 500-2000 J2 per well were used to quantify the amount of lectin bound to nematodes after addition of peroxidase substrate. Lectins were most likely bound to the surface of the Meloidogyne spp. J2 examined, since microscopic observation of lectin-treated J2 from aliquots transferred to peroxidase substrate reaction plates indicated that J2 were intact and viable. Different amounts of lectin bound to J2 of Mi1, Mi3, and Mj (Table 3-1). Preinfective J2 of Mj bound more SBA, LOT, and WGA than Mi1 or Mi3 J2, and preinfective J2 of Mi1 bound more LPA than Mj and Mi3 in two experiments. Populations of Mi3 bound less lectin than J2 of Mi1 and Mj in all tests except experiment 2 with LOT. Preinfective J2 of Mi1 bound the most CON A in experiment 1, and J2 of Mj bound the most CON A in experiment 2. Considerable differences in the relative amount of lectin which bound to J2 within a single lectin-nematode combination were detected between experiment 1 and experiment 2.

Table 3-1. Binding of peroxidase-labeled lectins to second-stage juveniles (J2) of Meloidogyne incognita race 1 (Mi1), M. incognita race 3 (Mi3), and M. javanica (Mj) as determined by microfiltration assay in two separate experiments.

Lectin	Picograms lectin/J2[a]		
	Mj	Mi1	Mi3
SBA[b]			
Exp. 1	4.20 ± 0.35[c]	1.36 ± 0.07	0.63 ± 0.03
Exp. 2	3.63 ± 0.21	1.30 ± 0.09	0.55 ± 0.03
CON A			
Exp. 1	0.66 ± 0.04	0.81 ± 0.03	0.62 ± 0.04
Exp. 2	1.04 ± 0.07	0.86 ± 0.06	0.70 ± 0.04
LOT			
Exp. 1	9.07 ± 1.01	4.79 ± 0.35	4.40 ± 0.49
Exp. 2	6.18 ± 0.43	4.17 ± 0.15	4.33 ± 0.13
WGA			
Exp. 1	3.42 ± 0.25	0.39 ± 0.11	0.21 ± 0.03
Exp. 2	1.92 ± 0.10	0.78 ± 0.07	0.54 ± 0.07
LPA			
Exp. 1	1.18 ± 0.05	2.31 ± 0.07	0.65 ± 0.05
Exp. 2	1.25 ± 0.07	3.13 ± 0.13	0.83 ± 0.05

[a] Nanograms lectin divided by the number of J2 (ca. 500-2000 J2) estimated for each sample.

[b] Soybean agglutinin (SBA); Concanavalin A (CON A); Lotus tetragonolobus agglutinin (LOT); wheat germ agglutinin (WGA); Limulus polyphemus agglutinin (LPA).

[c] Mean of 20 observations ± standard error.

Discussion

Differences in lectin binding among populations of Meloidogyne spp. that were not detected in assays with fluorescent lectins were detected by microfiltration enzyme-linked lectin assay. Variability in estimation of the number of J2 per sample most likely influenced the quantitative differences in lectin binding to J2 determined among Mi1, Mi3, and Mj and between experiments 1 and 2. If this were a major influence, however, standard errors should have been greater than those calculated for each experiment. The quantitative differences in lectin binding detected in these experiments could have been due to the production of carbohydrates in amphidial secretions of J2 of Meloidogyne spp. as reported above and elsewhere (76). Differences between experiments 1 and 2 may be due to the handling and relative age of the groups of J2 used in separate experiments. The rate of production of amphidial secretions by J2 of Meloidogyne spp. and the amount of amphidial secretion lost (if any) through the initial centrifugation or microfiltration wash remains unknown. Loss of excess amphidial secretion was noted in preliminary lectin-TRITC assays when centrifugation was used for all nematode washes, but this occurrence was inconsistent (unpublished results).

The technique described here for quantifying lectin binding to Meloidogyne spp. is useful because it is rapid and provides ease of handling compared to techniques used previously to quantify lectin binding to nematodes and bacteria (77, 88). The assay is very sensitive (nanogram level), but a considerable number of nematodes (several hundred) was necessary to achieve measurements above background levels. The potential use of this technique for quantification of lectin binding to other microorganisms or detection of nematode surface antigens by immunoassay should be considered.

CHAPTER 5
REPRODUCTION OF LECTIN-TREATED *MELOIDOGYNE* SPP. IN TWO RELATED SOYBEAN CULTIVARS

Introduction

Interactions between nematodes and other organisms are influenced by chemosensory stimuli (22, 29, 40, 45, 86, 131, 136). Researchers have postulated that intervention in host finding and recognition of nematodes may be achieved by blockage or obliteration of carbohydrates on nematode surfaces (135, 136). Proteins (lectins) that bind mannose, glucose, and sialic acids, and enzymes (glycohydrolases) which may cleave these carbohydrates from nematode surfaces impaired nematode chemotaxis toward source attractants (49, 53). Adhesion of conidia of *Meria coniospora* Drechs. to nematode chemosensory organs, nematode attraction to the fungus, and infection of nematodes by adhering conidia were inhibited by sialic acids, sialidase, or limulin (50, 51, 52). A lectin specific for mannose appeared to inhibit chemoreception necessary for the feeding and sexual attraction of males of *Trichostrongylus columbriformis* Giles (14). Capture of nematodes by *Arthrobotrys oligospora* Fres. appeared to involve interaction of lectin on fungal traps

with N-acetylgalactosamine moieties present on the nematode surface (17, 81).

Other studies have reported the presence of carbohydrates on the head region and surface of some phytoparasitic nematodes (see Chapter 2 above, 36, 76, 77, 95, 113, 115). Various lectins bound to carbohydrates present in amphidial exudates of second-stage juveniles (J2) of potato cyst and root-knot nematodes (see Chapter 2 above, 36, 76). Differences in structure and relative amounts of specific carbohydrates in amphidial carbohydrate complexes of several populations of Meloidogyne spp. have been reported above in Chapters 2 and 4.

Nematode surface carbohydrates may be involved in plant-nematode interactions (see Chapter 3 above, 74). Soil applications of Concanavalin A (Con A; mannose and glucose-specific lectin), and relatively higher concentrations of Limax flavus agglutinin (LFA; sialic acid-specific lectin), significantly suppressed galling of tomato roots induced by Meloidogyne incognita (Kofoid & White) Chitwood (74). Treatment of J2 from a population of race 3 of M. incognita with various lectins and carbohydrates promoted hypersensitivity in an apparently compatible soybean-M. incognita interaction (see Chapter 2 above). Nematodes could not be detected within soybean roots after inoculation of root tips with J2 of Meloidogyne spp. suspended in

solutions of LFA or sialic acid. The objective of this research was to determine the quantitative effect of several lectins and carbohydrates on establishment and reproduction of three populations of Meloidogyne spp. in two related soybean (Glycine max (L.) Merr.) cultivars.

Materials and Methods

Populations of Meloidogyne incognita races 1 and 3 (Mi1 and Mi3) and M. javanica (Treub) Chitwood (Mj) were maintained in greenhouse culture on roots of 'Rutgers' tomato (Lycopersicon esculentum Mill.) and 'Black Beauty' eggplant (Solanum melongena L.). Meloidogyne spp. populations were identified by adult female perineal patterns, second-stage juvenile (J2) lengths, and performance on differential hosts (101). Species identifications were also confirmed by three independent nematode taxonomists (A. M. Golden, Beltsville, MD; J. G. Baldwin, Riverside, CA; J. D. Eisenback, Blacksburg, VA). Eggs of each nematode population were extracted from host roots with 0.53% NaOCl for 30 seconds (46) and hatched at room temperature on a Baermann funnel. Preinfective J2 which had hatched within 48 hours were used as test organisms in each experiment.

Surface carbohydrates of Meloidogyne spp. J2 were blocked by incubating nematodes in solutions containing

unconjugated, purified soybean agglutinin (SBA), wheat germ agglutinin (WGA), Lotus tetragonolobus agglutinin (LOT), CON A, or Limulus polyphemus agglutinin (LPA) (E-Y Labs, San Mateo, CA). The sugar specificity of each lectin, corresponding competitive sugars, and procedure used to determine the specific hemagglutination activity for each lectin were described in Chapter 2. Buffer solutions included: 0.01 M phosphate-buffer saline (PBS) at pH 7.2 for SBA, WGA, and LOT; 0.05 M Tris-saline plus 0.01 M $CaCl_2$ at pH 7.5 for CON A; 0.05 M Tris-saline plus 0.01 M $CaCl_2$ at pH 8.0 for LPA.

Preinfective J2 of Mi1, Mi3, and Mj were concentrated in the appropriate buffer or in distilled water by centrifugation at 1000g for 3 minutes. Treatments for each lectin included incubating J2 (approx. 2000 J2) of each population in lectin solution (200 µg/ml), lectin (200 µg/ml) plus 0.1M competitive sugar, and 0.1M sugar solution minus lectin for 2 hours at 4°C. Control treatments included J2 in buffer and J2 in distilled water incubated for 2 hours at 4°C. Suspensions of J2 in each treatment (1.0 ml total volume per treatment) were diluted to 16 ml (12.5 µg/ml lectin and/or 6.25mM sugar) immediately before being added to soil in which soybeans were grown as described below.

Since a 0.1 M solution of sialic acid in 0.05 M Tris-saline buffer was quite acidic (pH ~ 3.0), a soybean root penetration bioassay was conducted to evaluate the effects of sialic acid neutralized with NaOH (pH 7.0) and of an acidic buffer (pH 3.0) on activity of J2 of Meloidogyne spp. (see Appendix D below). Solutions containing J2 of Mi1, Mi3, and Mj were incubated for 2 hours at 4°C, diluted 1:16 with Tris-saline buffer, placed on soybean roots, and the number of J2 within roots was determined after 24 hours.

Two related cultivars of soybean (Glycine max cv. Pickett 71 and Centennial) were used for root challenge by J2 of Meloidogyne spp. Pickett 71 was compatible and Centennial was incompatible with M. incognita, and both soybean cultivars were compatible with M. javanica (61). Individual soybean seedlings were grown in a greenhouse in 150-cm^3 Conetainers (Leach Nursery, Canby, OR) containing steam-pasteurized Astatula fine sand (hyperthermic, uncoated typic quartzipsamments). Two-milliliter suspensions (approx. 2000 J2) of each treatment combination were added to the soil in each Conetainer using a syringe fitted with a 10-cm-long canulus (24). There were four replicates of each treatment combination. Test plants were maintained in a glasshouse at 27 ± 3°C, watered daily, and fertilized once a week with a solution containing 10-6-10 (N-P-K) plus

microelements. Experiment 1 was conducted in the spring and experiment 2 was conducted in the summer.

Soybean plants were removed from Conetainers 60 days after soil was infested with J2 and the roots were rinsed free of soil. The number of Meloidogyne spp. egg masses per root system was rated on a 0-5 scale (101). Data were subjected to analysis of variance procedure and treatment differences were determined by the Waller-Duncan k-ratio t-test with k=100 ($P \leq 0.05$). This experiment was repeated once.

Results

Hemagglutination assays indicated that the binding capacity of pure lectins was relatively strong, except for LPA. Specific hemagglutination activities of 4096, 4096, 8192, 8192, and 16 units/mg lectin were determined for SBA, CON A, WGA, LOT, and LPA, respectively. Hemagglutination activity of all lectins was completely inhibited in the presence of 100 mM corresponding competitive sugar.

Pickett 71 soybean was highly compatible with Mi1 in two experiments, as indicated by the high egg mass ratings in buffer and water controls (Table 4-1). Little reduction in Mi 1 reproduction in Pickett 71 compared to controls was demonstrated by any lectin and sugar treatment except LPA plus sialic acid and sialic acid alone in the second test. This was not, however, verified by the results of the first

Table 4-1. Reproduction of Meloidogyne incognita race 1 in 'Pickett 71' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	4.50 a*	3.50 abcde	3.75 abcd	4.50 a	3.00 cde
Lectin + sugar	4.25 ab	3.50 abcde	3.00 cde	3.75 abcd	2.75 de
Sugar	3.25 bcde	3.75 abcd	3.25 bcde	4.25 ab	2.50 e
Buffer	4.00 abc	3.50 abcde	3.50 abcde	4.00 abc	2.50 e
Distilled water	4.00 abc	3.75 abcd	4.25 ab	4.25 ab	3.00 cde
Experiment 2					
Lectin	4.50 abc	4.25 bcd	4.50 abc	4.00 cd	4.50 abc
Lectin + sugar	4.25 bcd	4.75 ab	4.75 ab	5.00 a	1.00 e
Sugar	4.75 ab	3.75 d	4.50 abc	4.50 abc	3.75 d
Buffer	4.50 abc	4.00 cd	4.50 abc	5.00 a	4.75 ab
Distilled water	5.00 a	4.75 ab	4.25 bcd	4.75 ab	5.00 a

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

test. Reproduction of Mi1 on Pickett 71 in experiment 2 was significantly lower for LPA plus sialic acid compared to all other lectin plus sugar treatments. The rating for LPA plus sialic acid in the first experiment, however, was only significantly lower than that of SBA plus galactose. Treatment with LPA alone in the first experiment, significantly reduced reproduction of MI1 in Pickett 71 compared to SBA and CON A alone. Sialic acid significantly reduced the egg mass rating in experiment 1 compared to N-acetylglucosamine and mannose. Both buffer and water controls for LPA produced relatively low egg mass ratings, however, compared to all other treatments in experiment 1.

Centennial soybean was highly incompatible with Mi1, as indicated by poor nematode reproduction among all lectin, sugar, and control treatments (Table 4-2). Significantly lower egg mass ratings were produced in Centennial by Mi1 for several treatments in experiment 2; however the ratings were very low overall, and the few lower ratings in experiment 2 were not verified by results from the first experiment.

Race 3 of M. incognita was highly compatible with Pickett 71; almost all treatments and controls had high egg mass ratings (Table 4-3). Treatment with sialic acid alone and sialic acid plus LPA significantly reduced reproduction of Mi3 in Pickett 71 over that in the controls in both

Table 4-2. Reproduction of Meloidogyne incognita race 1 in 'Centennial' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	1.00 a*	0.50 a	0.75 a	0.75 a	0.75 a
Lectin + sugar	1.50 a	1.00 a	1.25 a	1.25 a	0.50 a
Sugar	1.75 a	1.25 a	1.00 a	1.00 a	0.50 a
Buffer	1.25 a	1.25 a	0.75 a	1.00 a	1.00 a
Distilled water	1.25 a	1.50 a	1.25 a	1.75 a	1.25 a
Experiment 2					
Lectin	1.25 abcde	1.75 abc	1.75 abc	1.00 bcde	1.50 abcd
Lectin + sugar	1.50 abcd	1.75 abc	1.25 abcde	1.50 abcd	0.75 cde
Sugar	1.75 abc	1.25 abcde	1.50 abcd	1.25 abcde	0.25 e
Buffer	1.50 abcd	2.00 ab	1.50 abcd	1.75 abc	1.50 abcd
Distilled water	2.25 a	1.50 abcd	1.75 abc	1.25 abcde	0.50 de

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

Table 4-3. Reproduction of Meloidogyne incognita race 3 in 'Pickett 71' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	4.75 ab*	4.75 ab	4.75 ab	5.00 a	4.00 bcd
Lectin + sugar	4.50 abc	3.75 cd	4.50 abc	3.25 de	2.50 ef
Sugar	5.00 a	3.75 cd	5.00 a	4.50 abc	2.25 f
Buffer	4.50 abc	4.00 bcd	4.75 ab	4.75 ab	4.75 ab
Distilled water	4.75 ab	4.75 ab	4.75 ab	4.50 abc	4.50 abc
Experiment 2					
Lectin	4.50 ab	4.25 bc	5.00 a	4.50 ab	4.50 ab
Lectin + sugar	5.00 a	4.75 ab	4.50 ab	4.50 ab	1.25 d
Sugar	4.75 ab	4.50 ab	5.00 a	4.75 ab	3.75 c
Buffer	5.00 a	4.75 ab	4.75 ab	5.00 a	4.50 ab
Distilled water	5.00 a	4.75 ab	5.00 a	5.00 a	5.00 a

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

experiments. Moderate reduction in Mi3 reproduction in Pickett 71 was observed for CON A plus mannose treatment, as compared to the controls, in experiment 1 but not in experiment 2.

Centennial soybean was relatively incompatible with Mi3, as indicated by low egg mass ratings for the majority of treatments in two experiments (Table 4-4). Significant differences in Mi3 reproduction in Centennial were observed for some treatments, but ratings were low overall and none of the reductions in one of the tests was verified by a similar reduction in the other test.

Pickett 71 soybean was compatible with Mj, as indicated by high egg mass ratings for many lectin, sugar, and control treatments (Table 4-5). Reproduction of Mj in Pickett 71 was significantly reduced by sialic acid and sialic acid plus LPA treatment compared to all other treatments in the second experiment. Treatment with sialic acid alone and LPA plus sialic acid significantly reduced reproduction of Mj in Pickett 71 compared to all other sugar, and lectin plus sugar treatments, respectively, in experiment 1. Ratings for sialic acid and LPA plus sialic acid were not significantly lower, however, than those for buffer and water controls of LPA in the first experiment.

Relatively high egg mass ratings for many lectin, sugar, and control treatments indicate that Centennial

Table 4-4. Reproduction of Meloidogyne incognita race 3 in 'Centennial' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	1.00 c*	1.00 c	1.00 c	2.00 abc	2.00 abc
Lectin + sugar	1.00 c	2.00 abc	1.25 bc	2.25 ab	1.25 bc
Sugar	1.50 abc	1.75 abc	1.25 bc	1.75 abc	1.50 abc
Buffer	2.50 a	1.50 abc	1.00 c	2.25 ab	1.75 abc
Distilled water	2.25 ab	1.75 abc	1.50 abc	1.25 bc	1.25 bc
Experiment 2					
Lectin	2.50 ab	1.75 bcd	2.00 abc	2.00 abc	1.50 cd
Lectin + sugar	2.75 a	2.50 ab	2.00 abc	2.50 ab	1.00 d
Sugar	2.50 ab	2.50 ab	2.25 abc	2.00 abc	1.75 bcd
Buffer	2.50 ab	2.75 a	2.00 abc	2.25 abc	2.75 a
Distilled water	2.75 a	2.50 ab	2.25 abc	1.75 bcd	2.50 ab

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

Table 4-5. Reproduction of Meloidogyne javanica in 'Pickett 71' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	3.75 ab*	2.50 cdefg	3.25 abcd	2.00 efg	2.75 bcdef
Lectin + sugar	3.00 abcde	3.75 ab	4.00 a	3.00 abcde	1.50 g
Sugar	3.00 abcde	3.25 abcd	3.75 ab	3.75 ab	1.75 fg
Buffer	3.25 abcd	3.50 abc	3.00 abcde	2.25 defg	2.00 efg
Distilled water	3.25 abcd	3.50 abc	2.25 defg	3.00 abcde	1.50 g
Experiment 2					
Lectin	4.00 bcd	4.50 abc	4.50 abc	3.00 e	4.25 abcd
Lectin + sugar	3.50 de	4.75 ab	4.75 ab	4.50 abc	1.25 f
Sugar	4.00 bcd	3.75 cde	5.00 a	4.50 abc	1.00 f
Buffer	4.00 bcd	4.25 abcd	4.25 abcd	4.50 abc	4.25 abcd
Distilled water	4.75 ab	4.50 abc	4.25 abcd	4.25 abcd	4.50 abc

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

soybean was strongly compatible with Mj (Table 4-6). Reproduction of Mj in Centennial was significantly reduced by sialic acid and LPA plus sialic acid compared to all other treatments in experiment 2. Treatment with LPA plus sialic acid significantly reduced egg mass ratings in the first experiment compared to SBA plus galactose and WGA plus N-acetylglucosamine. Reproduction of Mj in Centennial was significantly reduced by treatment with sialic acid in experiment 1, compared to egg mass ratings for galactose, N-acetylglucosamine, and fucose. Ratings for sialic acid alone and sialic acid plus LPA, however, were not significantly lower than those for buffer and water controls of LPA in the first experiment.

Discussion

Results from this research generally agree with the histological observations reported above in Chapter 3. The inhibition of reproduction of untreated Mi3 in Centennial soybean roots, however, contrasts with the apparently compatible response of Centennial root tissue to untreated Mi3 observed in histological tests. Intact giant cells were associated with untreated Mi3 in Centennial soybean roots 20 days after exposure of roots to infective J2 of Mi3. However, no gall formation or development of Mi3 past third-stage juvenile was observed 20 days after inoculation.

Table 4-6. Reproduction of Meloidogyne javanica in 'Centennial' soybean roots after treatment of second-stage juveniles with selected lectins and their competitive sugars.

Treatment	Egg mass rating[a]/lectin				
	SBA[b]	WGA	LOT	CON A	LPA
Experiment 1					
Lectin	4.50 ab*	3.00 cdef	3.75 abcd	2.25 fg	4.00 abc
Lectin + sugar	3.75 abcd	4.00 abc	3.00 cdef	2.75 defg	1.75 fg
Sugar	3.50 bcde	4.00 abc	4.25 ab	3.00 cdef	2.00 fg
Buffer	4.50 ab	4.75 a	4.50 ab	2.50 efg	3.00 cdef
Distilled water	3.50 bcde	3.50 bcde	4.50 ab	3.50 bcde	2.50 efg
Experiment 2					
Lectin	4.25 abc	4.50 abc	4.75 ab	4.00 bc	4.00 bc
Lectin + sugar	4.00 bc	4.75 ab	4.50 abc	4.00 bc	1.25 d
Sugar	4.25 abc	4.75 ab	4.25 abc	3.75 c	1.75 d
Buffer	4.75 ab	5.00 a	4.50 abc	4.75 ab	4.50 abc
Distilled water	4.75 ab	4.25 abc	4.75 ab	4.25 abc	4.00 bc

[a] Scale: 0 = 0; 1 = 1-2; 2 = 3-10; 3 = 11-30; 4 = 31-100; 5 = >100 egg masses/root system.

[b] Lectins and corresponding competitive sugars included soybean agglutinin (SBA) and galactose; wheat germ agglutinin (WGA) and N-acetylglucosamine; Lotus tetragonolobus agglutinin (LOT) and fucose; Concanavalin A (CON A) and mannose; Limulus polyphemus agglutinin (LPA) and sialic acid.

* Table values are the mean of four relicates. Means followed by the same letter for each experiment are not significantly different ($P \leq 0.05$) according the the Waller-Duncan k-ratio t-test.

Strong reduction in the rate of Mi3 development, or a possible nutritional deficiency which culminated in nematode death, may have occurred in this host-parasite relationship (91, 133) since no evidence of active plant defense (ie. hypersensitivity) occurred in histological tests. Differences in the degree of incompatibility of "M. incognita-resistant" soybean cultivars with several M. incognita populations have also been reported (105).

Any effect of lectin or sugar on successful nematode infection of soybean roots most likely occurred at initial infection; however, environmental conditions and duration of the experiment were conducive to at least two generations of root-knot nematode reproduction. One investigation has indicated that soil application of CON A significantly reduced galling of tomato roots by M. incognita (74), but the activity of CON A in soil was difficult to interpret. Although moderate reductions in egg mass ratings were occasionally associated with CON A, sialic acid appeared to have the greatest and most consistent adverse effect on successful nematode infection. These results are supported by the apparent inability of several Meloidogyne spp. J2 to penetrate soybean roots in "unwashed" sialic acid and LFA treatments (see Chapter 3 above). Hemagglutination tests determined that the binding capacity of LPA was relatively weak, and it was completely inhibited in the presence of 100

mM sialic acid. This may indicate that concentrations of LPA when mixed with sialic acid were insufficient to inhibit (and may have acted in combination with) the activity of sialic acid on root-knot nematode infection of soybean roots. Threshold levels of sialic acid that significantly inhibit nematode infection need to be determined. Microscopic observation of J2 treated with sialic acid and LPA, and penetration of soybean roots by J2 treated with sialic acid and LFA and "washed", indicated that these treatments are not lethal to J2 of Meloidogyne spp. (see Chapters 2 and 3 above). The inhibition of soybean root penetration after treatment of J2 of Meloidogyne spp. with sialic acid was apparently more than just an adverse effect of low pH (see Appendix D below). The adverse effect of sialic acids on Meloidogyne spp. reproduction in soybean may be manifested in impairment of host-finding and penetration by treated J2. Perhaps sialic acids act as "biological masks" similar to those found in other animal systems (104). Subsequent investigations of these phenomena may provide information valuable to the development of novel means of nematode management.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Proteins (lectins) which bind to specific carbohydrates were used as probes to characterize carbohydrates on the surfaces of second-stage juveniles (J2) of the root-knot nematodes Meloidogyne incognita races 1 and 3 (Mi1 and Mi3) and Meloidogyne javanica (Mj). The binding of the fluorescent (rhodamine conjugated) lectins, soybean agglutinin (SBA), Concanavalin A (CON A), wheat germ agglutinin (WGA), Lotus tetragonolobus agglutinin (LOT), and Limulus polyphemus agglutinin (LPA) to freshly-hatched, preinfective J2 of root-knot nematodes was comparable among the populations of Melodogyne spp. examined. It was apparent from these experiments that nematode viability was critical for accurate detection of fluorescent lectin binding to nematodes, and that rhodamine (TRITC) conjugates of lectin were preferable to fluorescein (FITC) conjugates since J2 of Meloidogyne spp. autofluoresced at the excitation wavelength of FITC. Viable, preinfective J2 bound fluorescent lectin almost exclusively in the vicinity of the amphidial (cephalic chemosensillae) openings. Substances to which lectins bound were concentrated and

sometimes emanated from the amphidial region of J2 of Meloidogyne spp. Amphids of J2 of Mi1. Mi3, and Mj labeled weakly with SBA, CON A, and LPA and strongly with WGA and LOT. The greater intensity of fluorescent labeling by WGA and LOT conjugates may have been due to their relatively higher binding capacities as indicated by hemagglutination assays. The presence of fucosyl and sialyl residues in amphidial secretions was supported by the inhibition of LOT and LPA binding, respectively, in the presence of 0.1 M corresponding competitive sugars. The binding of SBA, CON A, and WGA was not inhibited in the presence of 0.1 M corresponding competitive sugars. This probably indicates nonspecific binding of the lectins to nematodes, or possibly a strong affinity of SBA, CON A,and WGA for carbohydrate-specific sites in amphidial secretions. Different molecular forms of the competitive sugars chosen may have provided greater inhibition of lectin binding activity. Soybean agglutinin, CON A, and WGA may have bound to hydrophobic sites (ie. lipids) on the nematode surface, and would not have been influenced by competitive sugars. All fluorescent lectins tested bound to egg shells of Mi1, Mi3, and Mj and were not inhibited in the presence of competitive sugars.

Results from treatment of J2 with enzymes (glycohydrolases) that cleave specific carbohydrate residues from glycoconjugates, including α-galactosidase, α-

mannosidase, ß-N-acetylglucosaminidase, α-fucosidase, and sialidase (neuraminidase), and subsequent binding of fluorescent lectins to treated J2 suggested that differences in the sequence and spatial arrangement of amphidial carbohydrate complexes exist among Mi1, Mi3, and Mj. A number of different glycohydrolases eliminated binding of LPA-TRITC to amphids of J2 which suggested that sialic acids were some of the outermost carbohydrate moieties present in J2 amphidial carbohydrate complexes. Several glycohydrolases promoted binding of CON A to the anterior cuticle of Mi1 and Mj , and WGA to the anterior cuticle of Mi1.

Quantitative differences in lectin binding to J2 of Mi1, Mi3, and Mj were determined by a modified microfiltration enzyme immunoassay developed for use with peroxidase-labeled lectins instead of antibody probes. Preinfective J2 of Mj bound the greatest amount of SBA, LOT, and WGA while J2 of Mi1 bound the most LPA in two separate experiments. Preinfective J2 of Mi3 consistently bound the least amount of all lectins tested. This may indicate that preformed carbohydrates in amphidial secretions of J2 differ quantitatively, as well as in configuration, among different populations of Meloidogyne spp. The rate of production of amphidial secretions by J2 of Meloidogyne spp. has not been determined. The microfiltration assay using peroxidase-

labeled lectins was rapid and relatively easy to conduct. Although the assay was sensitive (nanogram level), it required relatively high numbers (approx. 2000) of J2 per sample to achieve final levels of lectin binding that were sufficiently higher than background levels.

Differential lectin binding to the head region of preinfective J2 suggested that carbohydrates were concentrated in this portion of invasive juveniles. Since the anterior end of these nematodes appears to stimulate hypersensitive incompatible (resistant) plant responses, it was hypothesized that blocking of preformed amphidial carbohydrates with lectins might influence recognition and specificty in incompatible and compatible Meloidogyne spp.-soybean interactions. The response of root tissue was examined histologically 5 days after exposure of M. incognita-compatible 'Pickett 71' and M. incognita-incompatible 'Centennial' soybean roots to lectin and/or sugar-treated J2 of Mi1, Mi3, and Mj. Untreated J2 of all three root-knot nematode populations induced the formation of feeding sites (giant cells) in Pickett 71 soybean roots, and untreated J2 of Mi3 and Mj induced giant cell formation in Centennial soybean roots 5 days after inoculation. Giant cells were maintained in Centennial 20 days after inoculation with untreated J2 of Mi3, but no gall formation, hyperplasia of pericycle cells adjacent to giant cells, or

development of Mi3 past third-stage juvenile was observed at day 20. Untreated J2 of Mi1 induced a hypersensitive response (HR; localized plant tissue necrosis) in Centennial soybean roots 5 days after inoculation. Treatment of J2 of Mi1 and Mj with SBA, CON A, WGA, LOT, LPA and/or their corresponding competitive sugars did not influence the root tissue response of either soybean cultivar to infection by these nematodes. Hence, the surface carbohydrates of Mi1 and Mj did not appear to be involved in plant-nematode interactions, but the fate of lectin bound to J2 once they have entered the plant root remains unknown. Treatment of J2 of Mi3 with any lectin and/or sugar tested, however, induced the formation of HR in Centennial soybean root tissue 5 days after inoculation. Treatment of J2 of Mi3 in this manner may actually have facilitated recognition of invasive J2 and subsequent defense response by the plant. The lack of specificty of lectin or sugar effects in the Mi3-Centennial interaction, however, makes it seem unlikely that alteration of surface carbohydrate composition of preinfective J2 and recognition by a carbohydrate-specific plant receptor was responsible for promoting incompatibility. If one considers the interaction of nematode surface carbohydrates with potential plant cell surface receptors as a "lock and key" phenomenon, however, it may be feasible that a slight alteration in surface

carbohydrate compostion was sufficient to promote incompatibility to Mi3 in Centennial soybean roots. Possibly a greater alteration of the carbohydrates examined here on Mi1 and Mj, or alteration of surface carbohydrates not examined in these studies, would influence their host-parasite interactions.

Treatment of J2 of Mi3 with lectin or sugar may have stimulated the production of a substance by the nematode that induces HR in Centennial soybean roots. Juveniles of Mi1 may inherently have the capacity to induce HR while J2 of Mj cannot promote incompatibility in soybean no matter what the treatment. For some populations, such as in the case of Mi3, incompatibility may be a process that can be stimulated. Conversely, substances produced by J2 of Meloidogyne spp. (ie. amphidial or stylet secretions) may be essential to induce compatibility between host and parasite, and these substances were altered sufficiently in Mi3 to inhibit compatibility in Centennial soybean roots.

The ability of J2 of Mi1, Mi3, and Mj to penetrate the roots of either soybean cultivar was apparently strongly impaired when J2 were introduced to roots in a solution that contained Limax flavus agglutinin (LFA; sialic acid-specific), sialic acid, or combination of the two. The inhibition of soybean root penetration after sialic acid treatment of J2 of Meloidogyne spp. was apparently more than

just an adverse effect of low pH. These treatments were not lethal to J2 of Meloidogyne spp. since J2 that were treated with LFA and/or sialic acid and rinsed regained their infectivity to both soybean cultivars. In addition, J2 treated with LPA-TRITC and/or sialic acid and rinsed were viable when observed microscopically.

The results of histological experiments were generally confirmed by greenhouse assays that were designed to quantify the effects of SBA, CON A, WGA, LOT, LPA, and their competitive sugars on Meloidogyne-soybean interactions. The reproduction of lectin and/or sugar-treated J2 of Mi1, Mi3, and Mj in Centennial and Pickett 71 soybean roots was determined by rating the number of root-knot nematode egg masses per root system 60 days after inoculation with treated J2. The reproduction of untreated Mi3 in Centennial soybean was extremely poor, in contrast to the apparent compatibility (giant cells) observed in Centennial root tissue after exposure of soybean roots to untreated J2 of Mi3. Only treatment of J2 of Mi1, Mi3, and Mj with sialic acid, and especially sialic acid plus LPA, significantly reduced reproduction of these nematodes in compatible soybean cultivars. This may have been an indirect result of reduced initial root penetration by J2 of Meloidogyne spp. that were exposed to these treatments. It is possible that sialic acid residues proximate to nematode

chemosensillae have a masking or regulatory effect similar to that observed in other animal systems. Modification of sialic residues in nematode chemosensory organs may strongly impair host finding and penetration of plant roots by root-knot nematodes.

APPENDIX A
BINDING OF FLUORESCENT SOYBEAN AGGLUTININ TO POSTINFECTIVE SECOND-STAGE JUVENILES OF *MELOIDOGYNE* SPP.

Alteration of nematode surface or secretory carbohydrates may influence plant compatibility with phytoparasitic nematodes. Changes in surface carbohydrates of second-stage juveniles (J2) of *Meloidogyne* spp. which may occur once J2 enter plant roots have never been studied. Nematode penetration and subsequent interaction with plant roots may stimulate or alter production of secretory or surface molecules by the nematode. Conversely, plant products such as lectins or carbohydrates may bind to sites which exist on infective J2 and influence plant-nematode interactions. We have conducted preliminary experiments to monitor potential changes in surface carbohydrates of J2 of several *Meloidogyne* spp. populations after they have entered roots of compatible and incompatible soybean cultivars.

Seeds of 'Pickett 71' and 'Centennial' soybean were germinated in ragdolls and placed on trays of autoclaved sand as described for histology experiments (see Chapter 3 above). One hundred-microliter suspensions (approx. 2000 J2 hatched within 48 hours of inoculation) of *Meloidogyne incognita* races 1 and 3 (Mi1, Mi3), and *M. javanica* (Mj) in

tap water were placed on separate soybean root tips of both cultivars. Soybean roots were washed free of sand and inoculated segments of roots were excised and placed in phosphate-buffer saline (PBS), pH 7.2, approximately 40 hours after inoculation.

Excised root segments of each Meloidogyne spp. population-soybean cultivar combination were immediately decorticated under a 40x dissecting microscope. Soybean root steles and cortices containing Meloidogyne spp. J2 were placed in separate BPI dishes containing PBS. The root tissue was incubated at room temperature overnight to allow J2 to emerge from the plant tissue. These "postinfective" J2 were washed three times with PBS and subsequently incubated in fluorescent, soybean agglutinin-tetramethylrhodamine isothiocyanate (SBA-TRITC) solution (200 µg/ml), 0.1 M D-galactose solution plus SBA-TRITC (200 µg/ml), 0.1M D-galactose solution minus SBA-TRITC, or distilled water for 2 hours at 4°C (see Chapter 2 above). Treated postinfective J2 were washed three times with PBS, mounted on glass slides, and immediately observed under epifluorescent microscopy.

Little difference in labeling of postinfective J2 with fluorescent SBA was observed among Meloidogyne spp. populations, soybean cultivars, and J2 from root cortices or steles. No fluorescence was observed on any postinfective

J2 treated with only 0.1M D-galactose or distilled water. Postinfective J2 of all Meloidogyne spp. populations tested labeled weakly with SBA-TRITC in the amphidial region, similar to results obtained with preinfective J2 (see Chapter 2 above). The cuticles of postinfective J2 of Mi1, Mi3, and Mj labeled strongly with SBA-TRITC, however, and this binding was greatly inhibited in the presence of 0.1M D-galactose. Binding of SBA-TRITC to cuticles of postinfective Meloidogyne spp. J2 was confined to the anterior half of the body of most nematodes observed (Fig. A-1). Fluorescent SBA did bind to the cuticle on the posterior half of some postinfective J2, but SBA-labeling was often discontinuous ("patchy") along the posterior cuticle (Fig. A-1).

The binding of SBA to cuticles of postinfective J2 of Meloidogyne spp. and the inhibition of SBA binding in the presence of D-galactose indicated the presence of galactosyl residues on the body wall of J2 that had penetrated soybean roots. The cuticles of preinfective J2 of Meloidogyne spp. did not bind SBA-TRITC in this manner. It is not known whether galactosyl residues on the body wall of postinfective J2 of Meloidogyne spp.are of plant or nematode origin.

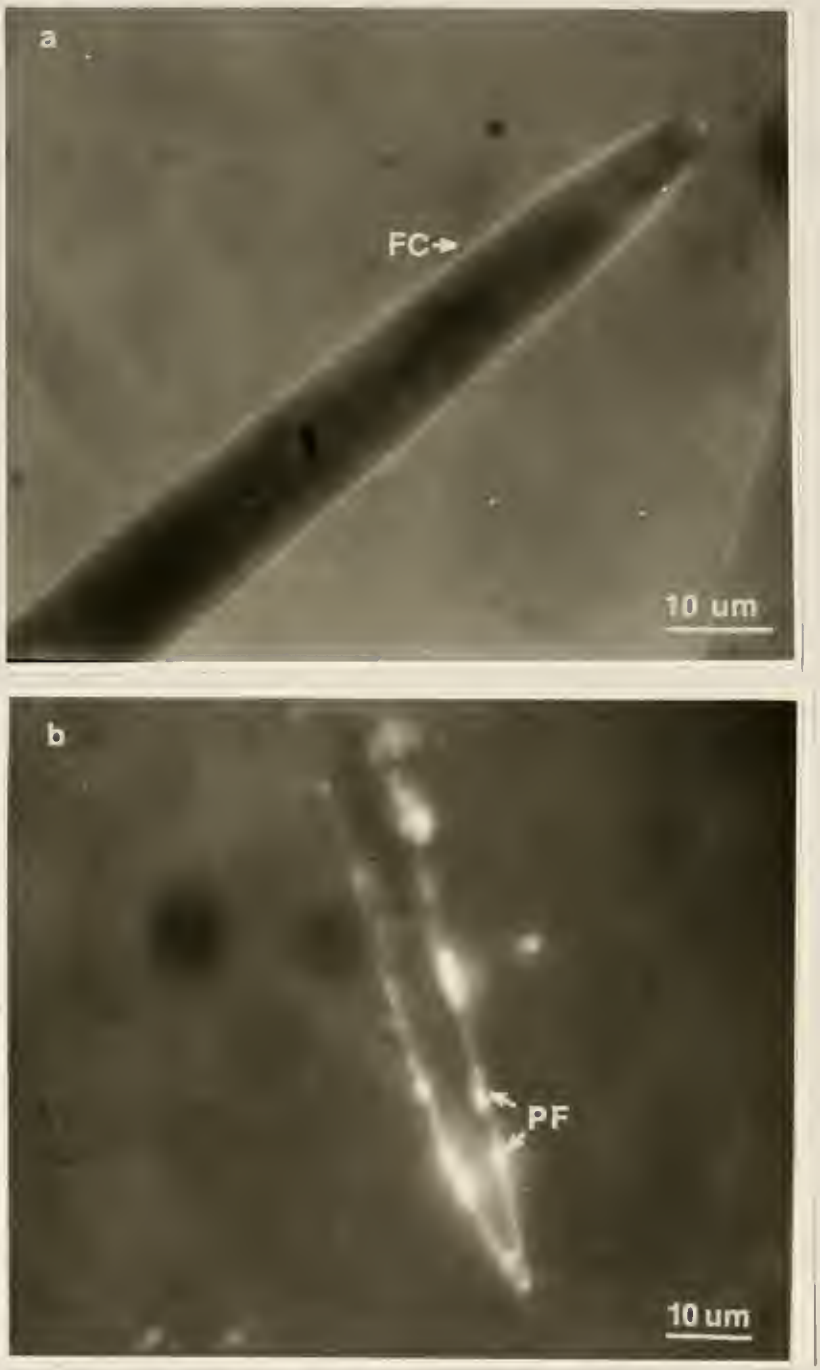


Fig. A-1. a) Binding of fluorescent (rhodamine conjugated) soybean agglutinin (SBA) to anterior cuticle of second-stage juvenile (J2) of Meloidogyne javanica that was removed from 'Centennial' soybean root stele; b) Discontinuous binding of fluorescent SBA to tail region of J2 of M. incognita race 3 that was removed from Centennial soybean root cortex. FC = fluorescent cuticle (note body annulation); PF = "patchy" fluorescence.

APPENDIX B
VIABILITY OF SECOND-STAGE JUVENILES OF *MELOIDOGYNE* SPP. AFTER EXPOSURE TO GLYCOHYDROLASE BUFFERS

The importance of nematode viability for accurate detection of *Meloidogyne* spp. surface carbohydrates with fluorescent lectins has been emphasized above in Chapter 2. The relatively low pH of most glycohydrolase buffers, the presence of ammonium sulfate in solution, and the incubation of second-stage juveniles (J2) in solutions at 37°C for extended periods of time may have threatened nematode viability in the glycohydrolase experiments presented above in Chapter 2. To determine the effect of experimental conditions (minus glycohydrolase) on viability of *Meloidogyne* spp. J2, the following bioassay was conducted.

Preinfective J2 of *Meloidogyne incognita* race 1 (Mi1) hatched within 48 hours were concentrated separately in the buffers described for glycohydrolase assays (see Chapter 2 above) or in distilled water. Treatments included Mi1 J2 incubated at 37°C for 2 hours and for 24 hours in one of the following solutions:

1. α-mannosidase buffer + 0.2M $(NH_4)_2SO_4$
2. α-galactosidase buffer

3. ß-N-acetylglucosaminidase buffer + 0.2M $(NH_4)_2SO_4$
4. α-fucosidase buffer + 0.2M $(NH_4)_2SO_4$
5. Neuraminidase (sialidase) buffer
6. α-mannosidase buffer + 0.2M $(NH_4)_2SO_4$ + 0.1M D-mannose
7. α-galactosidase buffer + 0.1M D-galactose
8. Distilled H_2O

Control treatments include Mi1 J2 in α-man buffer minus $(NH_4)_2SO_4$ or J2 in distilled H_2O at room temperature for 2 and 24 hours. Nematodes incubated in every treatment solution except distilled water appeared slightly vacuolated under a dissecting microscope.

Seedlings of 'Rutgers' tomato and 'California Wonder' pepper (5-10 cm tall) were grown in a greenhouse in small plastic cups containing Astatula fine sand. Suspensions of Mi1 J2 in incubation solutions were placed in the soil of individual tomato and pepper plants. Root systems were washed free of sand 10 days after inoculation with treated J2, and root systems were observed for the presence of nematode-induced galls.

Little difference in Mi1 infectivity was demonstrated among the treatments tested compared to untreated controls. The number of galls on pepper roots of all treatments was less than the number of galls observed on

tomato roots, but the relative amount of galling was consistent among treatments. The number of galls on tomato roots induced by J2 incubated in solutions 1 and 3 for 24 hours was slightly lower than controls. However, initial inoculum and gall number per root system were not quantified. Results of this assay indicate that the experimental conditions of the glycohydrolase assays above in Chapter 2 had little effect on viability of J2 of Meloidogyne spp.

APPENDIX C
BINDING OF FLUORESCENT LECTINS TO AXENIZED, PREINFECTIVE *MELOIDOGYNE* SPP.

Aseptic technique was not used in the fluorescent lectin assays presented above in Chapter 2. Since microbes, especially bacteria, produce extracellular carbohydrates that may label with fluorescent lectins, it was necessary to determine if microbial contaminants may have affected results reported above in Chapter 2. The following assay was conducted to observe fluorescent lectin binding to surface- disinfested (axenized) preinfective second-stage juveniles (J2) of *Meloidogyne* spp.

Second-stage juveniles (J2) of *Meloidogyne incognita* race 3 (Mi3) and *M. javanica* (Mj) were axenized according to the procedure of Krusberg and Sardanelli (67). Chromatography columns were packed with 2-6 mm diameter glass beads and filled with a solution containing 0.05% kanomycin sulfate and 0.01% chlorhexidine digluconate (Sigma Chemical Co., St. Louis, MO) in sterile tap water. Juveniles of each *Meloidogyne* spp. population that had hatched within 48 hours were placed in antibiotic solution at the top of separate columns. Nematodes migrated down the column overnight and were aseptically removed from the

bottom of the column the following morning. A small subsample of the axenized Meloidogyne spp. J2 was placed on potato dextrose agar (PDA) to check for microbial contamination. The remainder of the axenized J2 were immediately washed three times in appropriate lectin buffers and incubated for 2 hours at 4°C in the fluorescent lectin solutions described for preinfective Meloidogyne spp. J2 (see Chapter 2 above). Surface disinfested J2 treated with fluorescent lectin were washed, mounted on glass slides, and specimens were immediately observed under epifluorescent microscopy.

Binding of fluorescent lectins to axenized, preinfective J2 of Mi3 and Mj was almost identical to the results obtained with nonaxenized, preinfective J2 presented above in Chapter 2. The only exception was that the cuticle of almost all axenized J2 of Mi3 and Mj also labeled strongly with Concanavalin A (CON A) along the entire body surface. The binding of CON A to Meloidogyne spp. was not inhibited in the presence of 0.1M D-mannose. The reason for the binding of CON A to cuticles of axenized J2 of Meloidogyne spp. is unknown.

The subsample of axenized J2 of Mi3 and Mj that was placed on PDA proved negative for viable bacterial contamination. The presence or absence of dead bacteria on nematode surfaces was not confirmed. Results of this

axenizing assay suggest that active microbial contamination did not influence binding of fluorescent lectins to preinfective J2 of Meloidogyne spp. in experiments reported above in Chapter 2.

APPENDIX D
EFFECT OF ACIDITY OF SIALIC ACID ON PENETRATION OF SOYBEAN ROOTS BY SECOND-STAGE JUVENILES OF *MELOIDOGYNE* SPP.

Since a 100 mM solution of sialic acid in 50 mM Tris-saline resulted in a solution pH of approximately 3.0, the potential effect of low pH on soybean root penetration by second-stage juveniles (J2) of *Meloidogyne incognita* races 1 and 3 (Mi1, Mi3) and *M. javanica* (Mj) was bioassayed. Second-stage juveniles of Mi1, Mi3, and Mj were obtained and seeds of 'Pickett 71' soybean were germinated as described above in Chapter 3. Approximately 2000 J2 from each population of root-knot nematode were incubated for 2 hours at 4°C in 1.0 ml of either 100 mM sialic acid at pH 3.0, 100 mM sialic acid neutralized to pH 7.0 with NaOH, or 50 mM Tris-saline acidified to pH 3.0 with HCl. Approximately 32,000 J2 of each population were also incubated in identical solutions for 2 hours at 4°C, and the solution volume was increased from 1.0 ml to 16.0 ml with distilled water as described above in Chapter 5. Control treatments included aprroximately 2000 J2 incubated in 1.0 ml Tris-saline at pH 8.0 and approximately 2000 J2 incubated in 1.0 ml distilled water for 2 hours at 4°C.

One-hundred microliter suspensions of J2 (approx. 200 J2) in test solutions were applied to soybean roots growing on trays of autoclaved sand. Soybean root segments (1.0 cm long, including root tip) were excised 24 hours after inoculation with treated J2 and washed free of sand and external nematodes. Nematodes within excised roots were stained with acid fuchsin (20), and the number of J2 within each root segment was counted. Each treatment combination was replicated six times.

Both 100 mM sialic acid at pH 3.0 and 100 mM sialic acid at pH 3.0 diluted to 6.25 mM before inoculation caused a significantly greater reduction in penetration of soybean roots by J2 of Mi1, Mi3, and Mj than any other treatment (Table D-1). One hundred millimolar sialic acid neutralized to pH 7.0 with NaOH significantly reduced root penetration by J2 of Meloidogyne spp. compared to controls, but this effect was apparently lost when neutralized sialic acid was diluted to 6.25 mM. Undiluted 50 mM Tris-saline at pH 3.0 slightly reduced penetration of soybean roots by J2 of Mi1, Mi3 and Mj, but neither this treatment nor diluted Tris-saline at pH 3.0 significantly lowered J2 penetration of roots compared to controls. It seems apparent from this study that the adverse effect of 100 mM sialic acid on soybean root penetration by J2 of Meloidogyne spp. was more

Table D-1. Effect of 100 mM sialic acid at pH 3.0, 100 mM sialic acid neutralized to pH 7.0 with NaOH, and 50 mM Tris-saline at pH 3.0 on penetration of 'Pickett 71' soybean roots by second-stage juveniles (J2) of Meloidogyne spp.

Treatment	J2/root segment		
	Mj[a]	Mi1	Mi3
1) Sialic acid (100 mM, pH 3.0)	1.67 ± 0.96[b]	0.17 ± 0.15	1.67 ± 0.69
2) Sialic acid (100 mM, pH 7.0)	3.67 ± 1.22	5.50 ± 1.78	7.00 ± 1.03
3) Tris-saline (50 mM, pH 3.0)	9.00 ± 1.76	9.33 ± 2.09	13.50 ± 2.14
4) Sialic acid[c] (6.25 mM, pH 3.0)	1.00 ± 0.47	1.17 ± 0.60	0.67 ± 0.45
5) Sialic acid (6.25 mM, pH 7.0)	12.83 ± 1.83	13.67 ± 2.87	14.33 ± 1.80
6) Tris-saline (3.13 mM, pH 3.0)	12.33 ± 1.74	7.17 ± 1.56	11.00 ± 1.65
7) Tris-saline (50 mM, pH 8.0)	10.83 ± 1.92	6.67 ± 1.73	14.00 ± 2.40
8) Distilled water	12.17 ± 1.72	12.33 ± 1.49	18.67 ± 3.30

[a] Meloidogyne javanica (Mj) and M. incognita races 1 and 3 (Mi1 and Mi3).

[b] Mean of six observations ± standard error.

[c] Treatments 4, 5, and 6 were identical to treatments 1, 2, and 3, respectively, except that suspensions of J2 in incubation solutions were diluted from 1.0 to 16.0 ml to provide ca. 200 J2/100 µl for inoculation of soybean roots.

than just an effect of low pH, and that this effect is reduced when sialic acid solution is raised to pH 7.0 with NaOH.

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Eric L. Davis was born on March 18, 1958, in Long Branch, New Jersey. He attended Shore Regional High School in West Long Branch, New Jersey, and graduated in June, 1976. In September, 1976 he enrolled at the University of Rhode Island, Kingston, Rhode Island, and received a B.S. degree in plant science in June, 1980. He was chosen as the outstanding student in agronomy by the College of Resource Development and he also received the Presidential Student Excellence Award in Plant Science from the university president. In August, 1981 he enrolled in the University of Florida, Gainesville, Florida, as a graduate student in the Department of Entomology and Nematology. He received the M.S. degree in December, 1984 under the guidance of Dr. J. R. Rich, IFAS-AREC, Live Oak, Florida. His thesis was entitled "The role of nicotine in the resistance of tobacco to Meloidogyne incognita." In October, 1985 he moved to Orlando, Florida, to conduct reserach for this dissertation under the supervision of Dr. D. T. Kaplan, USDA-ARS, USHRL, Orlando, Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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